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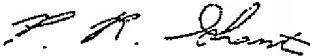
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Inventor(s)/Applicant(s)					
Last Name	First Name	Initial	Residence Address (City & either State or Foreign Country)		
BOYLE	WILLIAM	J.	11678 Chestnut Ridge Street, Moorpark, California 93021		
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Title of the Invention (250 characters max):					
TNF-RELATED PROTEINS					
Enclosed application parts are:					
<input checked="" type="checkbox"/> 48 pages of specification, 3 pages of claim(s), 1 pages of abstract, and 0 pages of sequence listing, totaling 52 pages					
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Respectfully submitted,					
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## TNF-RELATED PROTEINS

## Field of the Invention

The present invention relates to proteins that are involved in  
5 inflammation and immunomodulation, particularly in B cell growth,  
survival, or activation. The invention further relates to proteins related to  
the tumor necrosis factor (TNF)/nerve growth factor (NGF) superfamily  
and related nucleic acids, expression vectors, host cells, and binding  
assays. The specification also describes compositions and methods for the  
10 treatment of immune-related and inflammatory, autoimmune and other  
immune-related diseases or disorders, such as rheumatoid arthritis (RA),  
Crohn's disease (CD), lupus, and graft versus host disease (GvHD).

The invention also relates to receptors for these TNF-related  
proteins and methods and compositions for the treatment of inflammatory  
15 and immune-related diseases and disorders using the receptors.

## Background of the Invention

After years of study in necrosis of tumors, tumor necrosis factors  
(TNFs)  $\alpha$  and  $\beta$  were finally cloned in 1984. The ensuing years witnessed  
the emergence of a superfamily of TNF cytokines, including fas ligand  
20 (FasL), CD27 ligand (CD27L), CD30 ligand (CD30L), CD40 ligand  
(CD40L), TNF-related apoptosis-inducing ligand (TRAIL, also designated  
AGP-1), osteoprotegerin binding protein (OPG-BP or OPG ligand), 4-1BB  
ligand, LIGHT, APRIL, and TALL-1. Smith *et al.* (1994), *Cell* 76: 959-962;  
Lacey *et al.* (1998), *Cell* 93: 165-176; Chichepotiche *et al.* (1997), *J. Biol.*  
25 *Chem.* 272: 32401-32410; Mauri *et al.* (1998), *Immunity* 8: 21-30; Hahne *et*  
*al.* (1998), *J. Exp. Med.* 188: 1185-90; Shu *et al.* (1999), *J. Leukocyte Biology*  
65: 680-3. This family is unified by its structure, particularly at the C-  
terminus. In addition, most members known to date are expressed in  
immune compartments, although some members are also expressed in

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other tissues or organs, as well. Smith *et al.* (1994), *Cell* 76: 959-62. All ligand members, with the exception of LT- $\alpha$ , are type II transmembrane proteins, characterized by a conserved 150 amino acid region within C-terminal extracellular domain. Though restricted to only 20-25% identity, the conserved 150 amino acid domain folds into a characteristic  $\beta$ -pleated sheet sandwich and trimerizes. This conserved region can be proteolytically released, thus generating a soluble functional form. Banner *et al.* (1993), *Cell* 73: 431-445.

Many members within this ligand family are expressed in lymphoid enriched tissues and play important roles in the immune system development and modulation. Smith *et al.* (1994). For example, TNF $\alpha$  is mainly synthesized by macrophages and is an important mediator for inflammatory responses and immune defenses. Tracey & Cerami (1994), *Annu. Rev. Med.* 45: 491-503. Fas-L, predominantly expressed in activated T cell, modulates TCR-mediated apoptosis of thymocytes. Nagata, S. & Suda, T. (1995) *Immunology Today* 16: 39-43; Castrim *et al.* (1996), *Immunity* 5: 617-27. CD40L, also expressed by activated T cells, provides an essential signal for B cell survival, proliferation and immunoglobulin isotype switching. Noelle (1996), *Immunity* 4: 415-9.

The cognate receptors for most of the TNF ligand family members have been identified. These receptors share characteristic multiple cysteine-rich repeats within their extracellular domains, and do not possess catalytic motifs within cytoplasmic regions. Smith *et al.* (1994). The receptors signal through direct interactions with death domain proteins (e.g. TRADD, FADD, and RIP) or with the TRAF proteins (e.g. TRAF2, TRAF3, TRAF5, and TRAF6), triggering divergent and overlapping signaling pathways, e.g. apoptosis, NF- $\kappa$ B activation, or JNK activation. Wallach *et al.* (1999), *Annual Review of Immunology* 17: 331-67. These signaling events lead to cell death, proliferation, activation or differentiation. The expression profile of each receptor member varies.

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For example, TNFR1 is expressed on a broad spectrum of tissues and cells (13); whereas the cell surface receptor of OPGL is mainly restricted to the osteoclasts. Hsu et al. (1999) Proc. Natl. Acad. Sci. USA 96: 3540-5. It is therefore an object of the invention to identify proteins and nucleic acids  
5 related to TNFs. Such proteins are believed to play a role in inflammatory and immune processes, suggesting their usefulness in treating autoimmune and inflammatory disorders.

#### Summary of the Invention

In accordance with the present invention, the inventors describe a  
10 novel member of the tumor necrosis factor family. The novel TNF ligand family member is herein called TBAF (TNF family B cell Activation Factor) or AGP-3. Unlike other members of the family, the receptor for this member is primarily expressed in B cells, and its expression correlates to increases in the number of B cells and immunoglobulins produced. The  
15 natural, preferred human ortholog is here called AGP-3 or TBAF and contains 285 amino acids; the mouse ortholog, 309 amino acids. The protein is a type II transmembrane protein and has an amino terminal cytoplasmic domain, a transmembrane domain, and a carboxy terminal extracellular domain. TNF-related proteins of the invention may be  
20 membrane-associated or in soluble form, recombinantly produced or isolated after natural production. The present specification demonstrates that TBAF is a potent B cell stimulatory factor. Interestingly, the TBAF transgenic mice also developed autoantibodies and kidney immune complex deposits, a phenotype resembling lupus patients and lupus prone  
25 mice.

The invention provides for nucleic acids encoding such TNF-related proteins, vectors and host cells expressing the polypeptides, and methods for producing recombinant proteins. Antibodies or fragments thereof that specifically bind proteins are also provided.

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The subject proteins may be used in assays to identify cells and tissues that express AGP-3 or related proteins, and to identify new AGP-3-related proteins. Methods of identifying compounds that interact with AGP-3 protein are also provided. Such compounds include nucleic acids, peptides, proteins, carbohydrates, lipids or small molecular weight organic molecules and may act either as agonists or antagonists of AGP-3 protein activity.

AGP-3-related proteins are involved in B cell growth, survival, and activation, particularly in the lymph node, spleen, and Peyer's patches. AGP-3 agonists and antagonists thus modulate B cell response and may be used to treat diseases characterized by inflammatory processes or deregulated immune response, such as RA, GvHD, CD, lupus, and the like. Pharmaceutical compositions comprising AGP-3 proteins and AGP-3 agonists and antagonists are also encompassed by the invention.

#### Description of the Figures

Figure 1 shows the sequence of human AGP-3. Nucleic acid and amino acid sequences of human AGP-3 are indicated (SEQ ID NOS: 1 and 2, respectively). The predicted transmembrane region is underlined. Potential N-linked glycosylation sites are shown in boldface.

Figure 2 shows the sequence of murine AGP-3. Nucleic acid and amino acid sequences of murine AGP-3 are indicated (SEQ ID NOS: 3 and 4, respectively). The predicted transmembrane region is underlined. Potential N-linked glycosylation sites are shown in boldface.

Figure 3 shows an alignment of human and murine AGP-3, along with a consensus sequence (SEQ ID NO: 5). The predicted human and murine AGP-3 protein sequences were aligned by Pileup with gap creation penalty (12) and gap extension penalty (4) (Wisconsin GCG Package, Version 8.1, Genetics Computer Group Inc., Madison, Wisconsin). The consensus sequence was determined by Lineup

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(Wisconsin GCG Package, Version 8.1). The transmembrane regions from amino acid 47 to 72 in human AGP3 and from amino acid 48 to 73 in murine AGP3 are underlined. The N-terminal intracellular domain resides from amino acid 1 to 46 in human AGP3 and from amino acid 1 to 47 in murine AGP3. The C-terminal extracellular domain is localized from amino acid 73 to 285 in human AGP3, and from amino acid 74 to 309. The human and murine AGP-3 share 68% amino acid identity overall. The C-terminus of AGP-3 is more conserved between human and mouse, with 87% identity over a 142-amino acid length. The putative conserved beta strands are indicated at the top, with the amino acids forming the putative strands underlined.

Figure 4 shows human and murine AGP-3 mRNA tissue distribution. Human tissue northern blots (A) and murine tissue northern blots (B) were probed with <sup>32</sup>P-labeled human AGP-3 probe (A) or murine AGP-3 probe. The probed blots were exposed to Kodak film for 18 hours (A) or seven days (B).

Figure 5 shows histology analysis of AGP-3 transgenic mouse spleen. The spleen sections from control mouse (A, C and E) and AGP-3 transgenic mouse (B, D, and F) were stained with hematoxylin and eosin (A and B), anti-mouse B220 (C and D) or anti-mouse CD3 (E and F). The spleen of the transgenic mouse was enlarged, mainly due to the increase of size and number of the follicles. The B cell staining areas in the spleen follicles in the transgenic mouse were enlarged. The T cell number was slightly diminished.

Figure 6 shows histology analysis of AGP-3 transgenic mouse lymph nodes. The lymph node sections from control mouse (A, C and E) and AGP-3 transgenic mouse (B, D, and F) were stained with hematoxylin and eosin (A and B), anti-mouse B220 (C and D) or anti-mouse CD3 (E and F). The lymph node size of the transgenic mouse was enlarged. The B



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cell number was greatly increased in the transgenic mouse. Instead of restricted to marginal zones of the follicles as in the control mouse, the B cells also filled out the follicular area in the lymph nodes of the transgenic mouse. The T cell number was decreased in the transgenic mouse as compared to the control.

Figure 7 shows histology analysis of AGP-3 transgenic mouse Peyer's patches. The Peyer's patches sections from control mouse (A, C and E) and AGP-3 transgenic mouse (B, D, and F) were stained with hematoxylin and eosin (A and B), anti-mouse B220 (C and D) or anti-mouse CD3 (E and F). The histologic and immunohistologic changes were similar to the changes in the lymph node of the transgenic mouse.

Figure 8 shows FACS analysis of thymocytes, splenocytes and lymph node cells from AGP-3 transgenic mouse. Single-cell suspensions were prepared from spleen, lymph nodes and thymus from 10 AGP-3 transgenic mice and 5 control littermates. Cells were stained with FITC or PE-conjugated monoclonal antibodies against Thy-1.2, B220, CD11b, Gr-1, CD4 or CD8. The B cell population increased by 100% in the transgenic mice as compared to the control mice. The T cell population decreased approximately 36%, with similar reductions in both CD4+ and CD8+ populations. Similar changes, though to a lesser degree, were observed in splenocytes. No differences in thymocyte staining were observed between the transgenic or control group.

Figure 9 shows a sequence comparison of the C-terminal region of members of the TNF ligand family determined via Pileup (Wisconsin GCG Package, Version 8.1). Amino acid numbers are indicated on the left side. The putative conserved beta strands and loops are indicated at the top. The predicted N-glycosylation sites are indicated with asterisks. The top line shows the consensus sequence (SEQ ID NO: 6). The remaining lines

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show the sequence for the C-terminal region of the mammalian TNF-related protein identified (SEQ ID NOS: 7 to 24).

Figure 10 shows histology analysis of TBAF transgenic mice.

Sections of spleen (A, B, C), lymph node (D, E, F) and Payer's patches (G, H, D from control mice (left panel) and TBAF transgenic mice (right panel) were stained with hematoxylin and eosin (A, D, and G), anti-mouse B220 antibody (B, E, and H), or anti-mouse CD3 antibody (C, F, and I). Stained sections were analyzed under microscope at 10x.

Figure 11 shows FACS analysis splenocytes, lymph node cells and thymocytes of TBAF transgenic mice. Single-cell suspensions were prepared from spleen, lymph nodes and thymus from 10 TBAF transgenic mice and 5 control littermates. Cells, with stained with FITC or PE-conjugated monoclonal antibodies against thy-1.2, B220, CD11b, Gr-1, CD4 or CD8.

Figure 12 shows elevation of serum immunoglobulin levels in TBAF transgenic mice. Control mice (n=5) and TBAF transgenic mice (n=5) were bled successively at 6, 7, 8, 9, 11 and 12 weeks of age. Serum IgM, IgG, IgA, and IgE levels were quantitated by ELISA. Values are expressed as Mean  $\pm$  SEM. All TBAF immunoglobulin levels were significantly increased (T-test;  $P < 0.05$ ) compared to control groups.

Figure 13 shows kidney immunoglobulin deposits in TBAF transgenic mice. Kidney sections of 5 month control littermate (A, B, C), 5 month old TBAF mice (D, E, F), and 8 month old TBAF mice (G, H, I) were stained hematoxylin and eosin (A, D, and G), anti-mouse IgM (B, E, and H), anti-mouse IgG (C, F, and I), and Trichrome (G insert). Stained sections were analyzed under microscope at 60x.

Figure 14 shows that TBAF stimulates B cell survival and proliferation.

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A. Increased B cell viability in TBAF transgenic mice. B cells were isolated from spleens of 3 month old TBAF transgenic mice (n=3) and control littermates (n=3). A total of  $2.5 \times 10^5$  B cells was aliquoted per well in a 96-well round bottom plate and incubated for 9 days. At the indicated days, cells were incubated with  $5 \mu\text{g/ml}$  Propidium Iodide and subject to FACS analysis for positive staining cells. Values are expressed as Mean  $\pm$  SEM.

B. TBAF stimulates B cell proliferation. Purified B cells ( $10^5$ ) from B6 mice were cultured in triplicates in 96 well plate with indicated amount of TBAF at the absence (upper panel) or presence of  $2 \mu\text{g/ml}$  anti-IgM antibody (lower panel) for a period of 4 days. Proliferation was measured by radioactive  $^3\text{H}$  thymidine uptake in last 18 hours of pulse. Data shown represent mean  $\pm$  standard deviation of triplicate wells.

#### Detailed Description of the Invention

The following definitions apply to the terms used throughout this specification, unless otherwise limited in specific instances.

The term "AGP-3 related protein" refers to natural and recombinant proteins comprising the following sequence:

QDCLQLIADSTPTIXKGYTFVPWLLS?

(SEQ ID NO: 25)

wherein "X" may be any naturally occurring amino acid residue. This sequence is a consensus of the B and B'  $\beta$ -sheets and B/B' loop of hAGP3 and mAGP3 (see Figure 3), which is believed to be the specific receptor binding site. Preferred AGP-3 related proteins comprise both the B/B' consensus and the E/F consensus:

AMGHXIQRRVHVFGDELSLVTLFR

(SEQ ID NO: 26)

The E/F region is also believed to be involved in receptor binding. More preferred proteins are those comprising the consensus of the B-I region:

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QDCQLLIADŠ XTPTIXKGXY TFVFWLLSFK RGXALEEKEN KIXVXXTGYP  
PIYXQVLYTD XXXANGIXIQ RKKVEVEGDE LSLVTLPRCI QNMPXTLPNN  
SCYSAGIAXL EEGDEXQLAI PRENAQISXX GDXTFPGALK LL  
(SEQ ID NO: 27)

- 5 Most preferred proteins comprise the full C-terminal consensus sequence (SEQ ID NO: 5) or the B-I region of the human AGP-3 sequence (SEQ ID NO: 2). Such sequences can be included in naturally occurring proteins, truncated naturally occurring proteins, or recombinant and synthetic proteins. Recombinant and synthetic AGP-3 related proteins may be
- 10 formed by fusion of the AGP-3 derived fragment with unrelated molecules or molecular domains (e.g., Fc regions), domain swapping with other TNF family members (e.g., substitution of SEQ. ID. NO: 6 or NOS: 6 and 7 in a sequence derived from a different TNF family member), antibody grafting (e.g., substituting SEQ. ID. NO. 6 for an antibody CDR),
- 15 or other modifications. Such proteins are discussed further hereinbelow. The proteins may also be modified by linkage to a carbohydrate (e.g., dextran) or a water-soluble polymer (e.g., PEG). The proteins within this definition may also include substitution with amino acids serving as sites for attachment of non-protein groups (e.g., glycosylation sites). All such
- 20 proteins are encompassed by the term "AGP-3 related protein."

- "AGP-3 related activity" means that a natural or recombinant protein, analog, derivative or fragment is capable of modulating B cell growth, survival, or activation, particularly in MLN, spleen, and Peyer's patches. Such activity can be determined, for example, by such assays as
- 25 described in "Biological activity of AGP-3" in the Materials & Methods hereinafter, which may be modified as needed by many methods known to persons having ordinary skill in the art.

- An "analog" of an AGP-3 protein refers to a polypeptide within the definition of "AGP-3 related protein" that has a substitution or addition of
- 30 one or more amino acids such that the resulting polypeptide has at least

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the property of eliciting B cell growth, survival, or activation. Such analogs will have substitutions or additions at any place along the polypeptide. Preferred analogs include those of soluble AGP-3 related proteins. Fragments or analogs may be naturally occurring, such as a

5 polypeptide product of an allelic variant or a mRNA splice variant, or they may be constructed using techniques available to one skilled in the art for manipulating and synthesizing nucleic acids. The polypeptides may or may not have an amino terminal methionine residue.

A "derivative" of an AGP-3 protein is a polypeptide within the

10 definition of "AGP-3 related protein" that has undergone post-translational modifications. Such modifications include, for example, addition of N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends, attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked

15 carbohydrate chains, and addition of an N-terminal methionine residue due to prokaryotic host cell expression. In particular, chemically modified derivatives of AGP-3 related protein that provide additional advantages such as increased stability, longer circulating time, or decreased immunogenicity are contemplated. Of particular use is modification with

20 water soluble polymers, such as polyethylene glycol and derivatives thereof (see for example U.S. Patent No. 4,179,337). The chemical moieties for derivitization may be selected from water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The

25 polypeptides may be modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties. Polypeptides may also be modified at pre-determined positions in the polypeptide, such as at the amino terminus, or at a selected lysine or arginine residue within the

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polypeptide. Other chemical modifications provided include a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

5 The term "protein" refers to polypeptides regardless of length or origin, comprising molecules that are recombinantly produced or naturally occurring, full length or truncated, having a natural sequence or mutated sequence, with or without post-translational modification, whether produced in mammalian cells, bacterial cells, or any other expression system.

10 The invention provides for a protein referred to as an AGP-3 protein, which primarily acts on B cells. An EST bearing a portion of the AGP-3 sequence was obtained from a human fetal liver spleen cDNA library. A labeled cDNA fragment was used to probe a human spleen cDNA phage library (see "Cloning of Human AGP-3" in Materials &  
15 Methods hereinafter). The cDNA encoding a human AGP-3 was isolated from this phage library. The human protein is a type II transmembrane protein, having a short N-terminal intracellular region that differed from other members of the TNF ligand family and a long C-terminal extracellular region that comprises most of the conserved region of the  
20 TNF ligand family.

An EST encoding a murine ortholog was identified by BLAST search of Genbank using the human AGP-3 sequence. The corresponding cDNA clone was obtained from a mouse lymph node library and used to probe a mouse spleen cDNA phage library (see Materials & Methods  
25 hereinafter). The cDNA encoding a murine AGP-3 ortholog was isolated from this phage library.

Northern blots were used to determine tissue distribution of transcription of AGP-3 (see "Cloning of Murine AGP-3" in Materials & Methods hereinafter). In murine tissue, AGP-3 mRNA was detected

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mainly in spleen, lung, liver, and kidney. In human tissue, AGP-3 mRNA was detected predominantly in peripheral blood leukocytes, with weaker transcription in spleen, lung, and small intestine (see Figures 4A and 4B).

- The murine ortholog of AGP-3 was overexpressed in transgenic mice (see "Overexpression of murine AGP-3 in transgenic mice" in Materials & Methods hereinafter). In these transgenic mice, serum globulin and total protein levels increased greatly over control littermates while the albumin level remained the same (see "Biological activity of AGP-3" in Materials & Methods hereinafter). The mice also exhibited increases in the size and number of follicles in the spleen, lymph nodes, and Peyer's patches (Figures 5, 6, and 7). In their MLN, the mice exhibited 100% increases in the number of cells expressing CD45 receptor with concomitant decreases in cells expressing CD90, CD4, and CD8. These results correspond to an increase in the B cell population and a decrease in the T cell population in the MLN (Figures 6 and 8). Similar results were obtained in the spleen, but to a lesser extent (Figures 5 and 8).

#### Nucleic Acids

- The invention provides for isolated nucleic acids encoding AGP-3 related proteins. As used herein, the term nucleic acid comprises cDNA, genomic DNA, wholly or partially synthetic DNA, and RNA. These nucleic acids may be prepared or isolated as described in the working examples hereinafter or by nucleic acid hybridization thereof.

- Nucleic acid hybridization typically involves a multi-step process. A first hybridization step forms nucleic acid duplexes from single strands. A second hybridization step under more stringent conditions selectively retains nucleic acid duplexes having the desired homology. The conditions of the first hybridization step are generally not crucial, provided they are not of higher stringency than the second hybridization step. Generally, the second hybridization is carried out under conditions of high stringency,

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wherein "high stringency" conditions refers to conditions of temperature and salt that are about 12-20 °C below the melting temperature ( $T_m$ ) of a perfect hybrid of part or all of the complementary strands corresponding to Figure 1 (SEQ ID NO: 1) and Figure 2 (SEQ ID NO: 3). In one  
5 embodiment, "high stringency" conditions refer to conditions of about 65 °C and not more than about 1 M Na<sup>+</sup>. It is understood that salt concentration, temperature and/or length of incubation may be varied in either the first or second hybridization steps such that one obtains the hybridizing nucleic acid molecules according to the invention. Conditions  
10 for hybridization of nucleic acids and calculations of  $T_m$  for nucleic acid hybrids are described in Sambrook *et al.* (1989), Molecular Cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press, New York.

The nucleic acids of the invention may hybridize to part or all of the polypeptide coding regions of AGP-3 related proteins (e.g., SEQ ID NOS:  
15 2 and 4 as shown in Figures 1 and 2) and therefore may be truncations or extensions of the nucleic acid sequences shown therein. Truncated or extended nucleic acids are encompassed by the invention provided that the encoded proteins retain AGP-3 related activity. In one embodiment, the nucleic acid will encode a polypeptide of at least about 10 amino acids.  
20 In another embodiment, the nucleic acid will encode a polypeptide of at least about 20 amino acids. In yet another embodiment, the nucleic acid will encode a polypeptide of at least about 50 amino acids. The hybridizing nucleic acids may also include noncoding sequences located 5' and/or 3' to the coding regions for the AGP-3 related protein. Noncoding  
25 sequences include regulatory regions involved in expression of AGP-3 related protein, such as promoters, enhancer regions, translational initiation sites, transcription termination sites and the like.

In preferred embodiments, the nucleic acids of the invention encode mouse or human AGP-3 related protein. Most preferred are the



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nucleic acids encoding proteins of SEQ ID NOS: 2 and 4. Nucleic acids may encode a membrane-bound form of AGP-3 related protein or soluble forms that lack a functional transmembrane region. For human AGP-3 related protein, the predicted transmembrane region includes amino acid  
5 residues 47-72 inclusive as shown in Figure 1 (SEQ. ID. NO: 2); for murine AGP-3 related protein, residues 48-73 inclusive as shown in Figure 2 (SEQ ID NO: 4). Substitutions that replace hydrophobic amino acid residues in this region with neutral or hydrophilic amino acid residues would be expected to disrupt membrane association and result in soluble AGP-3  
10 related protein. In addition, deletions of part or all the transmembrane region would also be expected to produce soluble forms of AGP-3 related protein. Nucleic acids encoding SEQ ID NO: 5 as shown in Figure 3 or fragments and analogs thereof, encompass soluble AGP-3 related proteins.

Nucleic acid sequences of the invention may also be used for the  
15 detection of sequences encoding AGP-3 related protein in biological samples. In particular, the sequences may be used to screen cDNA and genomic libraries for related AGP-3 related protein sequences, especially those from other species. The nucleic acids are also useful for modulating levels of AGP-3 related protein by anti-sense technology or in vivo gene  
20 expression. Development of transgenic animals expressing AGP-3 related protein is useful for production of the polypeptide and for the study of in vivo biological activity.

#### Vectors and Host Cells

The nucleic acids of the invention will be linked with DNA  
25 sequences so as to express biologically active AGP-3 related protein. Sequences required for expression are known to those skilled in the art and include promoters and enhancer sequences for initiation of RNA synthesis, transcription termination sites, ribosome binding sites for the initiation of protein synthesis, and leader sequences for secretion.

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- Sequences directing expression and secretion of AGP-3 related protein may be homologous, i.e., the sequences are identical or similar to those sequences in the genome involved in AGP-3 related protein expression and secretion, or they may be heterologous. A variety of plasmid vectors
- 5 are available for expressing AGP-3 related protein in host cells (see, for example, Methods in Enzymology v. 185, Goeddel, D.V. ed., Academic Press (1990)). For expression in mammalian host cells, a preferred embodiment is plasmid pDSR $\alpha$  described in PCT Application No. 90/14363. For expression in bacterial host cells, preferred
- 10 embodiments include plasmids harboring the lux promoter (see co-owned and co-pending U.S. Serial No. 08/577,778, filed December 22, 1995). In addition, vectors are available for the tissue-specific expression of AGP-3 related protein in transgenic animals. Gene transfer vectors derived from retrovirus (RV), adenovirus (AdV), and adeno-associated virus (AAV)
- 15 may also be used for the expression of AGP-3 related protein in human cells for in vivo therapy (see PCT Application No. 86/00922).
- Prokaryotic and eukaryotic host cells expressing AGP-3 related protein are also provided by the invention. Host cells include bacterial, yeast, plant, insect or mammalian cells. AGP-3 related protein may also be
- 20 produced in transgenic animals, such as mice or goats. Plasmids and vectors containing the nucleic acids of the invention are introduced into appropriate host cells using transfection or transformation techniques known to one skilled in the art. Host cells may contain DNA sequences encoding AGP-3 related protein as shown in Figure 1, 2, or 3 or a portion thereof, such as the extracellular domain or the cytoplasmic domain.
- 25 Nucleic acids encoding AGP-3 related proteins may be modified by substitution of codons that allow for optimal expression in a given host. At least some of the codons may be so-called preference codons that do not alter the amino acid sequence and are frequently found in genes that

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are highly expressed. However, it is understood that codon alterations to optimize expression are not restricted to the introduction of preference codons. Examples of preferred mammalian host cells for AGP-3 related protein expression include, but are not limited to COS, CHOd-, 293 and

- 5 3T3 cells. A preferred bacterial host cell is Escherichia coli.

Polypeptides

The invention also provides AGP-3 related protein as the product of prokaryotic or eukaryotic expression of an exogenous DNA sequence. Exogenous DNA sequences include cDNA, genomic DNA and synthetic

- 10 DNA sequences. AGP-3 related protein may be the product of bacterial, yeast, plant, insect or mammalian cells expression, or from cell-free translation systems. AGP-3 related protein produced in bacterial cells will have an N-terminal methionine residue. The invention also provides for a process of producing AGP-3 related protein comprising growing  
15 prokaryotic or eukaryotic host cells transformed or transfected with nucleic acids encoding AGP-3 related protein and isolating polypeptide expression products of the nucleic acids.

- Polypeptides that are mammalian AGP-3 related proteins or are fragments, analogs or derivatives thereof are encompassed by the  
20 invention. In a preferred embodiment, the AGP-3 related protein is human AGP-3 related protein. A fragment of AGP-3 related protein refers to a polypeptide having a deletion of one or more amino acids such that the resulting polypeptide has at least the property of eliciting B cell growth, survival, or activation, especially in mesenteric lymph nodes. Said  
25 fragments will have deletions originating from the amino terminal end, the carboxy terminal end, and internal regions of the polypeptide. Fragments of AGP-3 related protein are at least about ten amino acids, at least about 20 amino acids, or at least about 50 amino acids in length. In preferred embodiments, AGP-3 related protein will have a deletion of one

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or more amino acids from the transmembrane region (amino acid residues 48-73 as shown in Figure 1), or, alternatively, one or more amino acids from the amino-terminus up to and/or including the transmembrane region (amino acid residues 1-73 as shown in Figure 1).

- 5 The invention further comprises AGP-3 related protein chimeras. Such proteins comprise part or all of an AGP-3 related protein amino acid sequence fused to a heterologous amino acid sequence. The heterologous sequence may be any sequence that allows the resulting fusion protein to retain AGP-3 related activity. In preferred embodiments, a heterologous
- 10 sequence is fused to a sequence comprising an AGP-3 related protein's B/B' region (SEQ ID NO: 25), the B/B' region and the E/F region (SEQ ID NO: 26) or the more complete B-I region (SEQ ID NO: 27). Such heterologous sequences include cytoplasmic domains that allow for alternative intracellular signaling events, sequences that promote
- 15 oligomerization (e.g., the Fc region of IgG), enzyme sequences that provide a label for the polypeptide, and sequences that provide affinity probes (e.g., an antigen-antibody recognition site).

- The polypeptides of the invention are isolated and purified from tissues and cell lines that express AGP-3 related protein, either extracted
- 20 from lysates or from conditioned growth medium, and from transformed host cells expressing AGP-3 related protein. Human AGP-3 related protein, or nucleic acids encoding same, may be isolated from human lymph node or fetal liver tissue. Isolated AGP-3 related protein is free from association with human proteins and other cell constituents.

- 25 A method for purification of AGP-3 related protein from natural sources (e.g. tissues and cell lines that normally express an AGP-3 related protein) and from transfected host cells is also encompassed by the invention. The purification process may employ one or more standard protein purification steps in an appropriate order to obtain purified

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protein. The chromatography steps can include ion exchange, gel filtration, hydrophobic interaction, reverse phase, chromatofocusing, affinity chromatography employing an anti-AGP-3 related protein antibody or biotin-streptavidin affinity complex and the like.

5     Antibodies

Antibodies specifically binding the polypeptides of the invention are also encompassed by the invention. The antibodies may be produced by immunization with full-length ACP-3 related protein, soluble forms of ACP-3 related protein, or a fragment thereof. The antibodies of the invention may be polyclonal or monoclonal, or may be recombinant antibodies, such as chimeric antibodies wherein the murine constant regions on light and heavy chains are replaced by human sequences, or CDR-grafted antibodies wherein only the complementarity determining regions are of murine origin. Antibodies of the invention may also be human antibodies prepared, for example, by immunization of transgenic animals capable of producing human antibodies (see, for example, PCT Application No. WO93/12227). The antibodies are useful for detecting ACP-3 related protein in biological samples, thereby allowing the identification of cells or tissues that produce the protein. In addition, antibodies that bind to ACP-3 related protein and block interaction with other binding compounds may have therapeutic use in modulating B cell growth, activation, and/or proliferation.

Antibodies to ACP-3 related proteins may be useful in treatment of immune-related diseases or conditions. Such antibodies may bind to ACP-3, preventing ACP-3 binding to its receptors, and are thus competitive inhibitors (i.e., having ACP-3 antagonistic activity). Antibodies can be tested for binding to ACP-3 related protein and examined for their ability to inhibit ACP-3-mediated B cell growth, survival, or activation associated

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with the disease or condition (see "Biological activity of AGP-3" in Materials & Methods hereinafter).

It is also anticipated that fragments, derivatives or analogs that lack AGP-3 agonistic activity will act as antagonists of the ligand-receptor interaction and inhibit ligand-mediated activity of the AGP-3 related protein. Such antagonists can be examined for their ability to inhibit AGP-3-mediated B cell growth, survival, or activation associated with the disease or condition (see "Biological activity of AGP-3" in Materials & Methods hereinafter).

10 Compositions

The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the AGP-3 related protein of the invention together with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant. The

15 invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of an AGP-3 related protein agonist or antagonist. The term "therapeutically effective amount" means an amount that provides a therapeutic effect for a specified condition and route of administration. The composition may be in a liquid or lyophilized form

20 and comprises one or more of the following:

- a diluent (e.g., Tris, acetate or phosphate buffers) having various pH values and ionic strengths;
- a solubilizer (e.g., Tween or Polysorbate);
- carriers (e.g., human serum albumin or gelatin);
- 25 • preservatives (e.g., thimerosal or benzyl alcohol); and
- antioxidants (e.g., ascorbic acid or sodium metabisulfite).

Selection of a particular composition will depend upon a number of factors, including the condition being treated, the route of administration and the pharmacokinetic parameters desired. A more extensive survey of

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component suitable for pharmaceutical compositions is found in  
Remington's Pharmaceutical Sciences (1980), 18th ed. (A. R. Gennaro, ed.)  
Mack, Easton, PA.

- In a preferred embodiment, compositions comprising soluble AGP-  
5 3 related proteins are provided. Also encompassed are compositions  
comprising soluble AGP-3 related protein modified with water soluble  
polymers to increase solubility, stability, plasma half-life and  
bioavailability. Compositions may also comprise incorporation of soluble  
AGP-3 related protein into liposomes, microemulsions, micelles or vesicles  
10 for controlled delivery over an extended period of time. Soluble AGP-3  
related protein may be formulated into microparticles suitable for  
pulmonary administration.

- Compositions of the invention may be administered by injection,  
either subcutaneous, intravenous or intramuscular, or by oral, nasal,  
15 pulmonary or rectal administration. The route of administration  
eventually chosen will depend upon a number of factors and may be  
ascertained by one of ordinary skill in the art.

- The invention also provides for pharmaceutical compositions  
comprising a therapeutically effective amount of the nucleic acids of the  
20 invention together with a pharmaceutically acceptable adjuvant. Nucleic  
acid compositions will be suitable for the delivery of part or all of the  
coding region of AGP-3 related protein and/or flanking regions to cells  
and tissues as part of an anti-sense therapy regimen.

#### Pharmaceutical Methods of Use

- 25 AGP-3 related protein and agonists or antagonists thereof may be  
used to treat conditions characterized by B cell growth, survival, and  
activation, such as autoimmune and inflammatory disorders. The  
invention also encompasses modulators (agonists and antagonists) of  
AGP-3 related protein and the methods for obtaining them. An AGP-3

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- related protein modulator may either increase or decrease at least one activity associated with AGP-3 related protein, such as ability to elicit B cell growth, survival, or activation in MLN, spleen, and Peyer's patches. Typically, an agonist or antagonist may be a co-factor, such as a protein, peptide, carbohydrate, lipid or small molecular weight molecule, that interacts with AGP-3 related protein to regulate its activity. Potential polypeptide antagonists include antibodies that react with either soluble or membrane-associated forms of AGP-3 related protein, and soluble forms of AGP-3 related protein that comprise part or all of the extracellular domain of AGP-3 related protein. Molecules that regulate AGP-3 related protein expression typically include nucleic acids that are complementary to nucleic acids encoding AGP-3 related protein and that act as anti-sense regulators of expression.

- AGP-3 and agonists thereof may be particularly useful in treatment of inflammatory conditions of the joints. Inflammatory conditions of a joint are chronic joint diseases that afflict and disable, to varying degrees, millions of people worldwide. Rheumatoid arthritis is a disease of articular joints in which the cartilage and bone are slowly eroded away by a proliferative, invasive connective tissue called pannus, which is derived from the synovial membrane. The disease may involve peri-articular structures such as bursae, tendon sheaths and tendons as well as extra-articular tissues such as the subcutis, cardiovascular system, lungs, spleen, lymph nodes, skeletal muscles, nervous system (central and peripheral) and eyes (Silberberg (1985), Anderson's Pathology, Kissane (ed.), II:1828). Osteoarthritis is a common joint disease characterized by degenerative changes in articular cartilage and reactive proliferation of bone and cartilage around the joint. Osteoarthritis is a cell-mediated active process that may result from the inappropriate response of chondrocytes to catabolic and anabolic stimuli. Changes in some matrix molecules of



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articular cartilage reportedly occur in early osteoarthritis (Thonar et al. (1993), Rheumatic disease clinics of North America, Moskowitz (ed.), 19:635-657 and Shirumei et al. (1992), Arthritis Rheum., 35:1304-1308).

AGP-3 and agonists thereof are believed to be useful in the treatment of  
5 these and related conditions.

AGP-3 related protein and agonists or antagonists thereof may also be useful in treatment of a number of additional diseases and disorders, including acute pancreatitis; ALS; Alzheimer's disease; cachexia/anorexia; asthma; atherosclerosis; chronic fatigue syndrome, fever; diabetes (e.g.,  
10 insulin diabetes); glomerulonephritis; graft versus host rejection; hemorrhagic shock; hyperalgesia, inflammatory bowel disease; inflammatory conditions of a joint, including osteoarthritis, psoriatic arthritis and rheumatoid arthritis; ischemic injury, including cerebral ischemia (e.g., brain injury as a result of trauma, epilepsy, hemorrhage or  
15 stroke, each of which may lead to neurodegeneration); lung diseases (e.g., ARDS); multiple myeloma; multiple sclerosis; myelogenous (e.g., AML and CML) and other leukemias; myopathies (e.g., muscle protein metabolism, esp. in sepsis); osteoporosis; Parkinson's disease; pain; sleep disturbance; neurotoxicity (e.g., as induced by HIV); learning impairment;  
20 pre-term labor; psoriasis; reperfusion injury; septic shock; side effects from radiation therapy, temporal mandibular joint disease, tumor metastasis; or an inflammatory condition resulting from strain, sprain, cartilage damage, trauma, orthopedic surgery, infection or other disease processes.

Agonists and antagonists of AGP-3 related protein may be  
25 administered alone or in combination with a therapeutically effective amount of other drugs, including analgesic agents, disease-modifying anti-rheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), and any immune and/or inflammatory modulators. Thus,

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agonists and antagonists of AGP-3 related protein may be administered with:

- Modulators of other members of the TNF/TNF receptor family, including TNF antagonists, such as Enbrel™, sTNF-R1, and Avakine™.
- Nerve growth factor (NGF) modulators.
- IL-1 inhibitors, such as IL-1ra, IL-1 antibodies, solubilized IL-1 receptor, and the like.
- IL-6 inhibitors (e.g., antibodies to IL-6).
- IL-8 inhibitors (e.g., antibodies to IL-8).
- IL-18 inhibitors (e.g., IL-18 binding protein or IL-18 antibodies).
- Interleukin-1 converting enzyme (ICE) modulators.
- Transforming growth factor- $\beta$  (TGF- $\beta$ ), TGF- $\beta$  family members, and TGF- $\beta$  modulators.
- Fibroblast growth factors FGF-1 to FGF-10, and FGF modulators.
- Osteoprotegerin (OPG) and OPG analogues.
- PAF antagonists.
- Keratinocyte growth factor (KGF), KGF-related molecules, and KGF modulators.
- COX-2 inhibitors, such as Celebrex™ and Vioxx™.
- Prostaglandin analogs (e.g., E series prostaglandins).
- Matrix metalloproteinase (MMP) modulators.
- Nitric oxide synthase (NOS) modulators, including modulators of inducible NOS.
- Modulators of glucocorticoid receptor.
- Modulators of glutamate receptor.
- Modulators of lipopolysaccharide (LPS) levels.

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- Anti-cancer agents, including inhibitors of oncogenes (e.g., fos, jun) and interferons.
- Noradrenaline and modulators and mimetics thereof.

Assay Methods of Use

5 AGP-3 related proteins may be used in a variety of assays for detecting AGP-3 receptors, agonists, antagonists and characterizing interactions with AGP-3 related proteins. In general, the assay comprises incubating AGP-3 related protein under conditions that permit measurement of AGP-3 related activity as defined above. Qualitative or  
10 quantitative assays may be developed. Assays may also be used to identify new AGP-3 agonists or antagonists and AGP-3 related protein family members.

Binding of natural or synthesized receptor, agonist, or antagonist to AGP-3 related protein may be carried out in several formats, including  
15 cell-based binding assays, membrane binding assays, solution-phase assays and immunoassays. In general, trace levels of a labeled binding molecule are incubated with AGP-3 related protein samples for a specified period of time followed by measurement of bound molecule by filtration, electrochemiluminescent (ECL, ORIGEN system by IGEN), cell-based or  
20 immunoassays. Homogeneous assay technologies for radioactivity (SPA; Amersham) and time-resolved fluorescence (HTRF, Packard) can also be implemented. Binding is detected by labeling a binding molecule (e.g., an anti-AGP-3 antibody) with radioactive isotopes ( $^{125}\text{I}$ ,  $^{35}\text{S}$ ,  $^3\text{H}$ ), fluorescent dyes (fluorescein), lanthanide ( $\text{Eu}^{3+}$ ) chelates or cryptates, or bipyridyl-ruthenium ( $\text{Ru}^{2+}$ ) complexes. It is understood that the choice of a labeled  
25 probe will depend upon the detection system used. Alternatively, a binding molecule may be modified with an unlabeled epitope tag (e.g., biotin, peptides, His<sub>6</sub>, myc) and bound to proteins such as streptavidin,

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anti-peptide or anti-protein antibodies that have a detectable label as described above.

Binding molecules in such assays may be nucleic acids, proteins, peptides, carbohydrates, lipids or small molecular weight organic compounds. The binding molecule may be substantially purified or present in a crude mixture. The binding molecules may be further characterized by their ability to increase or decrease AGP-3 related protein activity in order to determine whether they act as an agonist or an antagonist.

10 In an alternative method, AGP-3 related protein may be assayed directly using polyclonal or monoclonal antibodies to AGP-3 related proteins in an immunoassay. Additional forms of AGP-3 related proteins containing epitope tags as described above may be used in solution and immunoassays.

15 AGP-3 related proteins are also useful for identification of intracellular proteins that interact with the cytoplasmic domain by a yeast two-hybrid screening process. As an example, hybrid constructs comprising DNA encoding the N-terminal 50 amino acids of an AGP-3 related protein fused to a yeast GAL4-DNA binding domain may be used  
20 as a two-hybrid bait plasmid. Positive clones emerging from the screening may be characterized further to identify interacting proteins. This information may help elucidate an intracellular signaling mechanism associated with AGP-3 related protein and provide intracellular targets for new drugs that modulate inflammatory and immune-related diseases and  
25 conditions.

A variety of assays may be used to measure the interaction of AGP-3 related protein and agonists, antagonists, or other ligands in vitro using purified proteins. These assays may be used to screen compounds for their ability to increase or decrease the rate or extent of binding to AGP-3

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- related protein. In one type of assay, AGP-3 related protein can be immobilized by attachment to the bottom of the wells of a microtiter plate. A radiolabeled binding molecule and a test molecule can then be added either one at a time (in either order) or simultaneously to the wells. After
- 5 incubation, the wells can be washed and counted using a scintillation counter for radioactivity to determine the extent of binding to AGP-3 related protein. Typically, molecules will be tested over a range of concentrations, and a series of control wells lacking one or more elements of the test assays can be used for accuracy in evaluation of the results. An
- 10 alternative to this method involves reversing the "positions" of the proteins; i.e., immobilizing a binding molecule to the microtiter plate wells, incubating with the test compound and radiolabeled AGP-3 related protein, and determining the extent of binding. See, for example, chapter 18 of Current Protocols in Molecular Biology (1995) (Ausubel et al., eds.),
- 15 John Wiley & Sons, New York, NY.

- As an alternative to radiolabeling, AGP-3 related protein or a binding molecule may be conjugated to biotin and the presence of biotinylated protein can then be detected using streptavidin linked to an enzyme, such as horseradish peroxidase (HRP) or alkaline phosphatase
- 20 (AP), that can be detected colorimetrically, or by fluorescent tagging of streptavidin. An antibody directed to AGP-3 related protein or a binding molecule that is conjugated to biotin may also be used and can be detected after incubation with enzyme-linked streptavidin linked to AP or HRP.

- AGP-3 related protein or a binding molecule may also be
- 25 immobilized by attachment to agarose beads, acrylic beads or other types of such inert substrates. The substrate-protein complex can be placed in a solution containing the complementary protein and the test compound; after incubation, the beads can be precipitated by centrifugation, and the amount of binding between AGP-3 related protein and a binding molecule

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can be assessed using the methods described above. Alternatively, the substrate-protein complex can be immobilized in a column and the test molecule and complementary molecule passed over the column.

- Formation of a complex between ACP-3 related protein and the binding molecule can then be assessed using any of the techniques set forth above (i.e., radiolabeling, antibody binding, and the like).
- 5

- Another useful in vitro assay is a surface plasmon resonance detector system, such as the Biacore assay system (Pharmacia, Piscataway, NJ). The Biacore system may be carried out using the manufacturer's protocol. This assay essentially involves covalent binding of either ACP-3 related protein or a binding molecule to a dextran-coated sensor chip that is located in a detector. The test compound and the other complementary protein can then be injected into the chamber containing the sensor chip either simultaneously or sequentially and the amount of complementary protein that binds can be assessed based on the change in molecular mass that is physically associated with the dextran-coated side of the of the sensor chip; the change in molecular mass can be measured by the detector system.
- 10
- 15

- In vitro assays such as those described above may be used advantageously to screen rapidly large numbers of compounds for effects on complex formation with ACP-3 related protein. The assays may be automated to screen compounds generated in phage display, synthetic peptide and chemical synthesis libraries.
- 20

- Compounds that increase or decrease complex formation of ACP-3 related protein and ACP-3 binding molecules may also be screened in cell culture using cells and cell lines bearing such ligands. Cells and cell lines may be obtained from any mammal, but preferably will be from human or other primate, canine, or rodent sources. Such cells may be enriched from other cell types by affinity chromatography using publicly available
- 25

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procedures. Attachment of AGP-3 related protein to such cells is evaluated in the presence or absence of test compounds and the extent of binding may be determined by, for example, flow cytometry using a biotinylated antibody to AGP-3 related protein. Cell culture assays may be used advantageously to further evaluate compounds that score positive in protein binding assays described above.

#### Description of Working Examples/Preferred Embodiments

The following examples are offered to more fully illustrate the invention, but are not construed as limiting the scope thereof.

#### 10 Materials and Methods

##### Cloning of Human AGP-3

A TNF family profile search of the Genbank dbEST data base was performed. Smith et al.(1994), Cell, 76: 959-62; Luethy et al.(1994), Protein Science, 3: 139-46. One human EST sequence (GenBank accession number T87299) was identified as a possible new member of the TNF ligand. The EST was obtained from human fetal liver spleen cDNA library (The WashU-Merck EST Project). The cDNA clone (115371 3') corresponding to the EST sequence was obtained from Genome Systems, Inc. (St. Louis, MO). The cDNA fragment was released from the pT7T3D vector with EcoRI and NotI digestion. The fragment was approximately 0.7 kb in length and was used for the subsequent full-length cloning.

The <sup>32</sup>P-dCTP-labeled T87299 cDNA fragment was used as a probe to screen a human spleen cDNA phage library (Stratagene, La Jolla, CA). Recombinant phages were plated onto E. coli strain XL1-blue at approximately 5 x 10<sup>4</sup> transformants per 150 mm LB plate. Nitrocellulose filters were lifted from these plates in duplicates. Filters were prehybridized in 5x SSC, 50% deionized formamide, 5x Denhardt's solution, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA for 2 hours at 42 °C. The filters were then hybridized in the same solution with

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the addition of 5 ng/ml of labeled probe at 42°C overnight. The filters were first washed in 2x SSC and 0.1% SDS for 10 minutes at RT twice, and then washed in 0.1x SSC and 0.1% SDS at 65 °C for 30 minutes twice. The filters were then exposed to autoradiography with intensifying screens at -

- 5 80 °C overnight. Positive hybridizing plaques were determined by aligning the duplicate filters, and then picked up for subsequent secondary or tertiary screening till single isolated positive plaque was obtained. From total of one million recombinant phage clones, 8 positive plaques were obtained.

- 10 The pBluescript phagemid was excised from phage using the ExAssist™/SOLR™ System according to the manufacturer's description (Stratagene, La Jolla, CA). The excised phagemids were plated onto freshly grown SOLR cells on LB/ampicillin plates and incubated overnight. Single bacteria colony was amplified in LB media containing 100 µg/ml  
15 ampicillin. The plasmid DNA was prepared and both strands of cDNA insert were sequenced.

- The human AGP-3 cDNA (clone 13-2) is 1.1 kb in length. It encodes a LORF of 285 amino acids. FASTA search of the SwissProt database with the predicted AGP-3 protein sequence indicated that it is mostly related to  
20 human TNFα with 25% identity in C-terminal 116 amino acid overlap. Like other TNF ligand family members, human AGP-3 protein is a type II transmembrane protein, containing a short N-terminal intracellular domain (amino acids 1-46), a hydrophobic transmembrane region (amino acids 47-68) following by a long C-terminal extracellular domain (amino  
25 acid 69-285). The C-terminal extracellular domain of AGP-3 contained most of the conserved region of the TNF ligand family. Smith *et al.* (1994), *Cell*, 76: 959-62.

#### Cloning of Murine AGP-3



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An EST sequence (Genebank accession number AA254047) encoding a potential murine AGP-3 ortholog was identified by BLAST search of Genebank dbEST database with human AGP-3 sequence. The corresponding cDNA clone (722549 5') from mouse lymph node library was obtained from Genome Systems, Inc. (St. Louis, MO). The clone contained a 0.9 kb cDNA insert which could be released by EcoRI and NotI digestion. The 0.9 kb cDNA fragment encodes an open reading frame of 96 amino acids which shares 87% identity with the corresponding C-terminal human AGP-3 polypeptide sequence. A 0.41 kb EcoRI-XmnI fragment, which contained 290 bp coding region and 120 bp 3' non-coding region, was used as probe to screening a mouse spleen cDNA phage library (Stratagene, La Jolla, CA) for full length murine AGP-3 cDNA as described above. From one million recombinant phage clones, 6 positive plaques were obtained. The phagemid was excised from phage as described above. The plasmid DNA was prepared and both strands of cDNA insert were sequenced. The murine AGP-3 cDNA (clone S6) encodes a polypeptide of 309 amino acids. Like its human ortholog, murine AGP-3 is also a type II transmembrane region, containing a short N-terminal intracellular domain (amino acid 1-46), a hydrophobic transmembrane region (amino acid 47-68) following by a long C-terminal extracellular domain (amino acid 69-285). The human and murine AGP-3 share 68% amino acid sequence identity overall. However, the C-terminal 142 amino acid sequences share 87% identity between the two species. Preceding the highly conserved C-terminus region, there is an insertion of 30 extra amino acids in the murine AGP-3. Four out of 7 positive phage plaques were independent clones, yet they all shared the same coding sequences.

Expression of human and murine AGP-3 mRNA

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Multiple human or murine tissue northern blots (Clontech, Palo Alto, CA) were probed with  $^{32}$ P-dCTP labeled human AGP-3 0.7kb EcoRI-NotI fragment or murine AGP-3 0.41kb EcoRI-XmnI fragment, respectively. The Northern blots were prehybridized in 5x SSC, 50% deionized formamide, 5xDenhardt's solution, 0.5% SDS, and 100  $\mu$ g/ml denatured salmon sperm DNA for 2 hours at 42 °C. The blots were then hybridized in the same solution with the addition of 5ng/ml of labeled probe at 42°C overnight. The filters were first washed in 2x SSC and 0.1% SDS for 10 minutes at RT twice, and then washed in 0.1x SSC and 0.1% SDS at 65 °C for 30 minutes twice. The blots were then exposed to autoradiography. The human tissue northern blot analysis with human AGP-3 probe under stringent conditions revealed predominant AGP-3 transcripts with a related molecular mass of 2.4kb in peripheral blood leukocytes (Figure 4A). Weaker expression was also detected in human spleen, lung and small intestine (Figure 4A). Among murine tissues analyzed, murine AGP-3 mRNA, with a relative molecular mass of 2kb, was mainly detected in spleen, lung, liver and kidney (Figure 4B).

Overexpression of murine AGP-3 in transgenic mice

Procedure 1. Murine AGP-3 cDNA clone S6 in pBluescript SK(-) in pBluescript was used as template to PCR the entire coding region. T3 primer

5' AAT TAA CCC TCA CTA AAG GG 3'

SEQ ID NO: 28

was used as 5' PCR primer. The 3' end PCR primer, which contained a XhoI site, was

5' TCT CCC TCG AGA TCA CGC ACT CCA GCA AGT GAG 3'

SEQ ID NO: 29

PCR reactions were carried in a volume of 50  $\mu$ l with 1 unit of vent DNA polymerase (New England Biolabs) in 20 mM Tris-HCl pH 8.8, 10 mM

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(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1% Triton-X100, 10 µM of each dNTP, 1 µM of each primer and 10 ng of murine AGP-3 cDNA template. Reactions were performed in 94 °C for 45 s, 55 °C for 55 s, and 72 °C for 2 minutes, for a total of 35 cycles. The PCR fragment created a XhoI site at 3' end after the AGP-3 coding region. The 1 kb PCR fragment was purified by electrophoresis, and digested with XbaI (present in the pBluescript MCS, 80 bp upstream of AGP-3 starting Methionine) and XhoI restriction enzymes. The XbaI-XhoI PCR fragment was cloned into expression vector under the control of the human β-actin promoter. Graham et al.(1997), *Nature Genetics* 17: 272-4; Ray et al.(1991), *Genes Dev.* 5: 2265-73. The PCR fragment was sequenced to ensure no mutation. The murine AGP-3 expression plasmid was purified through two rounds of CsCl density gradient centrifugation. The purified plasmid was digested with ClaI, and a 6 kb fragment containing murine AGP-3 transgene was purified by gel electrophoresis. The purified fragment was resuspended in 5 mM Tris, pH 7.4, 0.2 mM EDTA at 2 µg/ml concentration. Single-cell embryos from BDF1 x BDF1-bred mice were injected as described (WO97 /23614). Embryos were cultured overnight in a CO<sub>2</sub> incubator and 15-20 2-cell embryos were transferred to the oviducts of pseudopregnant CD1 female mice.

Following term pregnancy, 62 offspring were obtained from implantation of microinjected embryos. The offspring were screened by PCR amplification of the integrated transgene in genomic DNA samples. Ear pieces were digested in 20 µl ear buffer (20mM Tris, pH8.0, 10mM EDTA, 0.5% SDS, 500 µg/ml proteinase K) at 55°C overnight. The sample was diluted with 200 µl of TE, and 2µl of the ear sample was used for the PCR reaction. The 5' PCR primer

5' AAC AGG CTA TTT CTT CAT CTA CAG 3'

SEQ ID NO: 30

resided in the murine AGP-3 coding region. The 3' PCR primer

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5' CTC ATC AAT GTA TCT TAT CAT GTC T 3'

SEQ ID NO: 31

resided in the vector 3' to the murine AGP-3 transgene. The PCR reactions were carried in a volume of 50 µl with 0.5 unit of Tag DNA polymerase (Boehringer Mannheim, Indianapolis, IN) in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 10 µM of each dNTP, 1 µM of each primer and 2 µl of ear sample. The mixtures were first heated at 94 °C for 2 min, and the PCR reactions were performed in 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, for a total of 35 cycles. Of the 62 offspring, 10 were identified as PCR positive transgenic founders.

At 8 weeks of age, all ten transgenic founders (animal 3, 6, 9, 10, 13, 38, 40, 58, 59, and 62) and five controls (animal 7, 8, 11, 12 and 14) were sacrificed for necropsy and pathological analysis. Portions of spleen were removed, and total cellular RNA was isolated from the spleens of all the transgenic founders and negative controls using the Total RNA Extraction Kit (Qiagen Inc., Chatsworth, CA). The expression of the transgene was determined by RT-PCR. The cDNA was synthesized using the SuperScript™ Preamplification System according to the manufacturer's instructions (Gibco BRL, Gaithersburg, MD). The primer

5' CTC ATC AAT GTA TCT TAT CAT GTC T 3'

SEQ ID NO: 32

which was located in the expression vector sequence 3' to the AGP-3 transgene, was used to prime cDNA synthesis from the transgene transcripts. Ten µg total spleen RNA from transgenic founders and controls were incubated with 1 µM of primer at 70°C for 10 min, and placed on ice. The reaction was then supplemented with 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 10 µM of each dNTP, 0.1 mM DTT and 200 U SuperScript II RT. After incubation at 42 °C for 50 min, the

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reaction was stopped by heating at 72 °C for 15 min. Total RNA were digested by addition of 2 U RNase H and incubation at 57 °C for 20 min. Subsequent PCR reactions were carried out by using murine AGP-3 specific primers. The 5' PCR primer was

5' AGC CGC GGC CAC AGG AAC AG 3'

SEQ ID NO: 33

The 3' PCR primer was

5' TGG ATG ACA TGA CCC ATA G 3'

SEQ ID NO: 34

- 10 The PCR reaction was performed in a volume of 50 µl with 0.5 unit Tag DNA polymerase in 10 mM Tris-HCl pH 8.3, 50mM KCL, 2.5mM MgCl<sub>2</sub>, 10 µM of each dNTP, 1 µM of each primer and 1 µl of cDNA product. The reaction was performed at 94 °C for 30 s, 55°C for 30 S, and 72 °C for 1 min, for a total of 35 cycles. The PCR product was analyzed by
- 15 electrophoresis. Transgene expression was detected in the spleen of all ten AGP-3 transgenic mice founders.

Procedure 2. A XbaI-XhoI DNA fragment encoding murine full-length TBAF protein was cloned into expression vector under the control of the β-actin promoter. Graham *et al.* (1997), *Nature Genetics* 17: 273-4. A

- 20 6 kb ClaI fragment containing TBAF transgene was then injected to single-cell embryos from BDF1xBDF1-bred mice, and transgenic mice were generated as described. The transgene founders were determined by genomic DNA PCR with 5' primer:

AAC AGG CTA TTT CTT CAT CTA CAG

- 25 and 3' primer:

CTC ATC AAT GTA TCT TAT CAT GTC T

residing in the TBAF coding region or in transgenic vector 3' immediately after TBAF coding region, respectively. Transgene expression was

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subsequently determined by RT-PCR analysis with primers described above.

Biological activity of AGP-3

Prior to euthanasia, all animals from procedure 1 above were

- 5 weighed, anesthetized by isofluorane and blood was drawn by cardiac puncture. The samples were subjected to hematology and serum chemistry analysis. The serum globulin level in all the AGP-3 transgenic mice (animal 3, 6, 9, 10, 13, 38, 40, 58, 59 and 62) increased more than 100% as compared to the control littermates (animal 7, 8, 11, 12 and 14, Table 1).
- 10 Total protein level also increased correspondingly in the transgenic group, while albumin level remained the same. No significant differences in other serum chemistry or hematology parameters were observed at this age.

Radiography was performed after terminal exsanguination. There was no difference in the radiodensity or radiologic morphology of the

15 skeleton. Upon gross dissection, major visceral organs were subject to weight analysis. The spleen weight relative to the body weight increased by approximately 45% in the AGP-3 transgenic group as compared to the control mice. The sizes of lymph nodes and Peyer's patches were also increased substantially in all the AGP-3 transgenic mice.

- 20 Following gross dissection, tissues were removed and fixed in 10% buffered Zn-Formalin for histological examination. The tissues collected were liver, spleen, pancreas, stomach, the entire gastrointestinal tract, kidney, reproductive organs, skin and mammary glands, bone, brain, heart, lung, thymus, trachea, esophagus, thyroid, adrenals, urinary
- 25 bladder, lymph nodes and skeletal muscle. After fixation, the tissues were processed into paraffin blocks, and 3  $\mu$ m sections were obtained. All sections were stained with hematoxylin and eosin, and subject to histologic analysis. The size and the number of the follicles in the spleen, lymph nodes and Peyer's patches were increased significantly in the AGP-

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- 3 transgenic mice (Figure 5, 6 and 7). The spleen, lymph node and Peyer's patches of both the transgenic and the control mice were subject to immunohistology analysis with B cell and T cell specific antibodies. The formalin fixed paraffin embedded sections were deparaffinized and
- 5 hydrated to deionized water. The sections were quenched with 3% hydrogen peroxide, blocked with Protein Block (Lipshaw, Pittsburgh, PA), and incubated in rat monoclonal anti-mouse B220 and CD3 (Harlan, Indianapolis, IN), respectively. The binding was detected by biotinylated rabbit anti-rat immunoglobulins and peroxidase conjugated streptavidin
- 10 (BioGenex, San Ramon, CA) with DAB as chromagen (BioTek, Santa Barbara, CA). Sections were counterstained with hematoxylin. The B cell numbers, as indicated by positive B220 staining, increased significantly in the spleen, lymph nodes and Peyer's patches (Figure 5, 6, and 7). The T cell numbers, as indicated by the anti-CD3 staining, were slightly decreased.
- 15 There were no differences in the morphology of the thymus between the transgenic and the control group. By immunohistology, the T cell population was similar in numbers. At 8 weeks of age, there are no distinctive morphologic changes in the liver, kidneys, or urinary, central nervous, hematopoietic, skeletal, respiratory, gastrointestinal, endocrine,
- 20 or reproductive systems.

- After necropsy, MLN and sections of spleen and thymus from 10 AGP-3 transgenic mice (animals 3, 6, 9, 10, 13, 38, 40, 58, 59 and 62) and 5 control littermates (animals 7, 8, 11, 12, and 14) were removed. Single cell suspensions were prepared by gently grinding the tissues with the flat end
- 25 of a syringe against the bottom of a 100  $\mu$ m nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ). Cells were washed twice in a 15 ml volume then counted. Approximately 1 million cells from each tissue was stained with 0.5  $\mu$ g antibody in a 100  $\mu$ l volume of PBS (without Calcium and Magnesium) + 0.1% Bovine Albumin + 0.01% Sodium Azide. All

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spleen and MLN samples were incubated with 0.5 µg CD16/32(FcγIII/II) Fc block in a 20 µl volume for 10 minutes prior to the addition of FITC or PE-conjugated monoclonal antibodies against CD90.2 (Thy-1.2), CD45R (B220), CD11b(Mac-1), Gr-1, CD4, or CD8 (PharMingen, San Diego, CA) at 2-8 °C for 30 min. The cells were washed then analyzed by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA). Thymus samples were stained with FITC conjugated anti-Thy-1.2, FITC conjugated anti-CD4, and PE conjugated anti-CD8 (PharMingen, San Diego, CA).

In the MLN of the AGP-3 transgenic mice, the percentage of B220 positive B cells increased by 100% (Figure 6). The percentage of the Thy-1.2 positive T cells decreased approximately 36%, with similar reductions in both CD4(+) and CD8(+) populations. The helper CD4(+) / suppressor CD8(+) ratio remained unchanged. Similar increases in B cell and reductions in T cell populations were also observed in the spleens of the AGP-3 transgenic mice (Figure 8), though to a lesser extent. No obvious changes in staining with anti-CD11b or anti-Gr-1 antibodies were observed in the lymph node and spleen between the transgenic and the control group. In the thymus, there were no differences in the percentages of Thy-1.2(+), CD4(+), CD8(+) or CD4(+)CD8(+) populations between the AGP-3 transgenic and the control mice.

Histopathology, Immunostaining, and FACS Analysis of Transgenic Mice (procedure 2)

Following gross dissection, tissues were removed, fixed in 10% buffered Zn-formalin, processed into paraffin blocks, and 3 µm sections were obtained. All sections were stained with hematoxylin and eosin and subject to histologic analysis. For immunostaining, frozen sections were stained rat monoclonal anti-mouse B220 and CD3 (Harlan, Indianapolis, IN), respectively. The binding was detected by biotinylated rabbit anti-rat immunoglobulins and peroxidase conjugated streptavidin (BioGenex, San



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- Ramon, CA) with DAB as chromagen (BioTek, Santa Barbara, CA). For FACS analysis, single cell suspensions were prepared by gently grinding the tissues with the flat end of a syringe against the bottom of a 100 •m nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ). Cells were
- 5 washed twice in a 15 ml volume then counted. Approximately 1 million cells from each tissue was stained with 0.5 µg antibody in a 100 µl volume of PBS + 0.1% Bovine Albumin + 0.01% Sodium Azide. All spleen and MLN samples were incubated with 0.5 µg CD16/32(FcγIII/II) Fc block in a 20 µl volume for 10 minutes prior to the addition of FITC or PE-
- 10 conjugated monoclonal antibodies against CD90.2 (Thy-1.2), CD45R (B220), CD11b(Mac-1), Gr-1, CD4, or CD8 (PharMingen, San Diego, CA) at 2-8 °C for 30 min. The cells were washed then analyzed by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA). Thymus samples were stained with FITC conjugated anti-Thy-1.2, FITC conjugated anti-CD4, and
- 15 PE conjugated anti-CD8 (PharMingen, San Diego, CA).

Serum Immunoglobulin and Autoantibody Analysis

- Transgenic mice and control littermates from procedure 2 were bled successively at 6, 7, 8, 9, 11, and 12 weeks of age. Serum immunoglobulin levels were quantitated using by ELISA with Mouse
- 20 Hybridoma Subtype Kit as suggested by manufacture (Boehringer Mannheim, Indianapolis, IN). Presence of autoantibodies directed against nuclear antigens and dsDNA were examined in the serum by enzyme linked immunosorbant assay (ELISA). The levels of anti-nuclear antibodies were detected using ANA screen kit (Sigma) and anti-mouse
- 25 IgG peroxidase secondary antibody. Mouse serum samples were diluted 1:200 in ANA screen ELISA. For the detection of anti-dsDNA autoantibodies in serum, high binding ELISA plates were coated with plasmid DNA (Immunovision) as an antigen in the presence of methylated BSA. After blocking the non-specific sites and washing, diluted mouse

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serum samples were added to wells in duplicated and the binding was quantitated using horse radish peroxidase-labeled anti-mouse IgG or anti-mouse IgM reagents (Southern Biotech). A pooled positive serum from BWF1 mice and pooled negative serum from B6 mice was used as controls.

- 5 Experiment for the detection of anti-histone antibodies was essentially done similar to anti-DNA ELISA except that carbonate-bicarbonate buffer (pH9.6) buffer was used as coating buffer. Serum antibody data were compared by Mann Whitney test using Sigmastat software (SPSS Science, Chicago, IL).

10 B Cell Survival and Proliferation Assay

- Cells were isolated from spleens of 2-4 months old mice by negative selection. Briefly, B lymphocytes were purified by density gradient centrifugation and then passed over a B cells column (Accurate/Cedarlane, Westbury, NY). Cells isolated by this method were analyzed by flow cytometry and >90% were found positive for B220 staining.
- 15 Isolated B cells were cultured in MEM+10% FCS at 37°C, 5%CO<sub>2</sub>. Cells were collected from triplicate wells daily on day 1 through day 9 and incubated with 5 •g/ml Propidium Iodide. Cells were analyzed by Flow cytometry and the percentage of dead cells was calculated. For B cell proliferation assay, purified (10<sup>6</sup>) B cells from B6 mice as described above were cultured in MEM+10% heat inactivated FCS in triplicate in 96 well flat bottomed plate with/without 2 µg/ml of Goat F(ab')<sub>2</sub> anti-mouse IgM (Jackson ImmunoResearch Laboratory, West Grove, Pennsylvania) and/or indicated amount of recombinant TBAF for a period of 4 days at 37 °C,
- 20 5%CO<sub>2</sub>. Proliferation was measured by an uptake of radioactive <sup>3</sup>H) thymidine in last 18 hours of pulse. Data is shown in figure 14 as mean±standard deviation of triplicate wells.

Recombinant TBAF Protein

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Bacteria expression plasmids were constructed to express soluble human TBAF protein from amino acid 128 to 285 preceded by an artificial methionine start codon. *E. coli* were induced during fermentation, the lysate was applied to Q Sepharose FF (Pharmacia, Piscataway, NJ)

- 5 equilibrated in 10 mM Mes pH 6.0 and eluted with 50-400mM NaCl gradient over 30 column volumes. Fractions containing TBAF were pooled and loaded onto a Q Sepharose HP column (Pharmacia, Piscataway, NJ) equilibrated in 10 mM Tris-HCL pH 8.5. TBAF was eluted with an increasing linear NaCL gradient (50 mM-200 mM) over 30
- 10 column volumes. Endotoxin was removed by application to Sp HiTRAP column (Pharmacia, Piscataway, NJ) pH 4.8 and eluted with 100-500 mM NaCl in 10mM sodium acetate pH 4.8 over 25 column volumes. Final endotoxin level of the purified protein is approximately 0.2 EU/mg. The purified human TBAF is truncated at residue Arg133 as indicated by N-
- 15 terminal sequencing and has a molecular weight of 16.5 KDa by reducing SDS-PAGE.

#### Results

##### Cloning of TBAF, a novel TNF ligand family member

A TNF family profile search of the GeneBank dbEST database was performed.

- 20 One human EST sequence (GeneBank accession number T87299) was identified as a possible new member of the TNF ligand family. The full-length clone (AGP3) was subsequently isolated from screening a human spleen cDNA library. It encodes a LORF of 285 amino acids which is mostly related to human TNF with 25% identity in the C-terminal 116 amino acid overlap. This novel ligand
- 25 member was named as TBAF (TNF family B cell Activation Factor). Like most other TNF ligand family members, TBAF is a type II transmembrane protein. Among the tissues examined, human TBAF is predominantly expressed in peripheral blood leukocytes. Weaker expression was also detected spleen, lung, and small intestine. The mouse ortholog of TBAF was isolated from mouse

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spleen cDNA library. The human and murine TBAF share 68% amino acid sequence identity overall, whereas the C-terminal receptor-binding regions share 87% identity between the two species. TBAF is identical to the recently published TALL-1/BAFF/BlyS. Shu et al. (1999) J. Leukocyte Biology 65: 680-3; 5 Schneier et al. (1999), J. Exp. Med. 189: 1747-56; Moore et al. (1999), Science 285: 260-3.

B Cell Hyperplasia and Hypergammaglobulinemia in TBAF Transgenic Mice

To gain insights into the biological function for TBAF, transgenic 10 mice were generated that expressed full-length murine TBAF protein driven by the ubiquitous  $\alpha$ -actin promoter. Founder mice harboring the TBAF transgene were identified by PCR analysis of genomic DNA samples. Transgene expression was confirmed by RT-PCR from spleen total RNA. At 8 weeks of age, ten TBAF transgenic mice and five control 15 littermates were subject to necropsy and pathological analysis. The transgenic mice were of normal size and weight. However, the spleen weight relative to the body weight increased by approximately 45% in the TBAF transgenic group as compared to the control mice. The sizes of lymph nodes and Payer's Patches were also increased substantially in all 20 the TBAF transgenic mice. Histology analysis demonstrated that the size and the number of the follicles in the spleen, lymph nodes and Payer's patches were increased significantly in the TBAF transgenic group (Figure 10). Immunohistology staining with B and T cell specific markers indicated the B cell numbers increased significantly in the spleen, lymph 25 nodes and Payer's patches of the transgenic group (Figure 10). The T cell numbers, as indicated by the anti-CD3 staining, were decreased correspondingly (Figure 10). There were no differences in the morphology and immunostaining of thymus between the transgenic and the control groups. No changes were observed in other organs or organ

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systems of the 8 weeks old transgenic mice, including kidney, liver, and hematopoietic tissues.

The B cell hyperplasia phenotype in the TBAF transgenic mice was also confirmed by flow cytometry analysis. In the mesenteric lymph nodes of the TBAF transgenic mice, the percentage of B220 positive B cells increased by 100% (Figure 11). The percentage of the Thy-1.2 positive T cells decreased by approximately 36%, with similar reductions in both CD4(+) and CD8(+) T cells. Similar increase in B cell and reduction in T cell populations were also observed in the spleens of the TBAF transgenic mice, though to a lesser extent (Figure 11). Of note, the total T cell numbers in the lymph node and spleen of TBAF transgenic mice were similar to the control littermates. In the thymus, there were no differences in the percentages of single positive CD4(+) or CD8(+) T cells, or CD4(+)CD8(+) populations between the TBAF transgenic and the control mice (Figure 11). No obvious changes in staining with anti-CD11b or anti-Gr-1 antibodies were observed in the lymph nodes and spleen between the transgenic and the control group (Figure 11). The histological and FACS analysis, together, suggested severe B cell hyperplasia phenotype in the TBAF transgenic mice.

We also examined B cell populations of different developmental stages by FACS analysis. No differences were observed in the percentage of the pro B (B220+IgM-), immature B (B220+IgM+), or mature B (IgM+IgD+) within splenic B cell population of the TBAF transgenic mice as compared to the control littermates. In addition, the number of the splenic CD5+ B cells in the TBAF transgenic mice from 1 to 9 month of age was unaltered. We also didn't detect any alteration of the CD40 expression level on B cells in the transgenic mice, suggesting that the B cell hyperplasia in the TBAF transgenic mice was not caused by CD40 upregulation.

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In addition to the B cell hyperplasia phenotype, the TBAF transgenic mice also had severe hypergammaglobulinemia. The serum globulin level in TBAF transgenic mice increased more than 100% as compared to the control group. Total protein level also increased correspondingly in the transgenic, while albumin level remained the same. The increased B cell numbers and high serum globulin level suggested elevated serum immunoglobulin titer. Thus we examined serum levels of IgM, IgG, IgA and IgE of TBAF transgenic mice from 6 to 12 weeks of age. Comparing to the same age control littermates, serum IgM, IgG, IgA and IgE were significantly increased in all age groups of TBAF transgenic mice. The increase found in serum IgG was not specific to any particular subclass (IgG1, IgG2a, IgG2b, and IgG3). No significant differences in other serum chemistry or hematology parameters were observed at this age. The increased serum immunoglobulin levels is likely to result directly from increased B cell number, but may also be aggravated by increased B cell antibody production.

Autoantibodies associated with lupus in TBAF transgenic mice

Increased humoral immunity in TBAF transgenic mice warranted us to look for possible phenotypes resembling B cell associated autoimmune diseases such as systemic lupus erythematosus (SLE). The common denominator in lupus patients and lupus prone mice is IgG autoantibody production, and the hallmark of this disease is the presence of elevated anti-nuclear antibodies in the serum. The emergence of anti-DNA antibodies represents one final outcome in the different murine lupus models and patients with SLE. When sera from transgenic and non-transgenic mice at various age were examined for the presence of autoantibodies recognizing nuclear antigens or dsDNA, two different lines of TBAF transgenic mice began to show presence of autoantibodies at around 8 weeks of age (Table 1). The amount of anti-nuclear and anti-

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dsDNA antibody increased with their age in the transgenic animals (Table 1). More interestingly, at 5 and 8 months of age, TBAF transgenic mice showed 5-10 higher amount of anti-dsDNA antibodies compared to age matched lupus prone (NZBxNZW)F1 mice. The presence of

5 autoantibodies in the serum of TBAF transgenic mice did not discriminate between gender of mice. Both IgG and IgM antibodies to dsDNA were detected in transgenic animals. Presence of such autoantibodies was undetectable in non-transgenic littermates, as expected.

Immune Complex Deposits in the Kidney of TBAF Transgenic Mice

- 10 Presence of anti-DNA antibodies followed by immune complex induced renal damage is classical picture seen in lupus associated nephritis. At 5 month of age, the TBAF transgenic mice developed glomerular proteinaceous deposits in the kidney (Figure 13). The deposits were seen in more than 60% of the glomeruli in the transgenic mice, but
- 15 absent in the control littermates. Immunohistology showed the deposits contained moderate amounts of IgG and larger amounts of IgM (Figure 13). Trichrome staining showed no deposit of connective tissues in the glomeruli at 5 month of age. There is also no evidence of any cellular proliferation or presence of inflammatory cells at this age (Figure 13).
- 20 Interestingly, the kidney lesions progressed as the transgenic mice grew older. At 8 month of age, there was obvious enlargement of glomeruli in the TBAF transgenic mice as compared to the age matched control littermates (Figure 13G). In addition, we also detected extensive connective tissue deposits in the enlarged glomeruli (Figure 13G).
- 25 Comparing to the 5 month old mice, the 8 month old transgenic mice had increased IgG level in the glomeruli immune complex deposits (Figure 13I). Majority of the glomeruli in the TBAF transgenic mice were affected. We also performed serum and urine chemistry analysis of 5 month old and 8 month old TBAF transgenic along with the control littermates. No

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significant differences were noticed in the 5 month old TBAF transgenic mice. However, in the 8 month old mice, we observed increases in serum blood urea nitrogen (BUN) and calcium levels and decrease in serum phosphate level. In addition, the 8 month old TBAF mice also had increased protein level in the urine. These changes, together, suggest the onset of renal failure in the 8 month old TBAF transgenic mice. In conclusion, the high serum autoantibodies followed by the kidney lesions in the TBAF transgenic mice clearly resemble to the pathological progression in the SLE patients and lupus prone mice.

10     TBAF Stimulates B Cell Survival and Proliferation: a Possible  
          Mechanism for Autoimmunity

13     The B cell hyperplasia phenotype in the TBAF transgenic mice  
14     might arise from increased B cell survival and/or increased B cell  
15     proliferation. We first compared the viability of B cells from TBAF  
16     transgenic mice with that of the control littermates. B cells were isolated  
17     from both transgenic or control mice and incubated in minimal essential  
18     medium supplemented with 10% heat inactivated fetal bovine serum.  
19     Viability of the B cells was measured by FACS analysis for Propidium  
20     Iodide uptake (Figure 14A). By day 3, 30% of B cells isolated from the  
21     control mice were dead, whereas only 10% of B cells from TBAF  
22     transgenic mice were dead. By day 5, 70% of B cells from TBAF mice were  
23     still viable, whereas only 15% of B cell from control littermates were  
24     viable. By day 9, almost 50% of the TBAF transgenic B cells still remained  
25     viable. Therefore, transgenic expression of TBAF prolonged B cell  
viability. It remains to be determined if this B cell survival stimuli result  
directly from TBAF action on B cells or through its modulation of the  
immune system.

Recently Schneider et al (Schneider et al., 1999, and Moore et al., 1999) reported co-stimulation of B cell proliferation by BAFF/BLYS with



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anti-IgM. We found that TBAF alone can also stimulates B cell proliferation in a dose dependent manner with an ED<sub>50</sub> of approximately 3ng/ml (Figure 14B, upper). A ten fold increase of B cell proliferation was detected by TBAF treatment at 10 ng/ml concentration as compared to the untreated cells. In our experiment, anti-IgM alone at 2 ug/ml concentration increased B cell proliferation by 24 fold. Treatment with anti-IgM (2 ug/ml) in combination with various doses of TBAF led to dose dependent increase of B cell proliferation, with a maximal 13 fold increase as compared anti-IgM treatment alone and a total of 320 fold increase as compared to the untreated cells. Thus, TBAF is a potent B cell stimulatory factor. The increased B cell survival and proliferation may together contribute to the B cell hyperplasia and autoimmune lupus like changes in the TBAF transgenic mice.

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Table 1: Lupus associated autoantibodies in the serum of TBAF transgenic mice.

Autoantibodies	Age (months)	TBAF Ig (n)	Non-Ig littermates (n)	p value
Antinuclear antibodies (IgG) <sup>a</sup>	2-3	7 <sup>*</sup> (9)	1 <sup>*</sup> (8)	
	5-6	9 (9)	1 <sup>*</sup> (8)	
	8-9	8 (8)	1 <sup>*</sup> (6)	
Anti-dsDNA (IgG) <sup>a</sup>	<2	697±284 (7)	277±67 (7)	NS
	3-4	842±351 (7)	235±49 (7)	<.005
	6-7	2515±428 (5)	970±344 (7)	<.019
	8-10	12293±6767 (11)	1070±602 (12)	<.017
Anti-dsDNA (IgM) <sup>a</sup>	<2	275±33 (7)	46±5 (7)	<.001
	3-4	1684±920 (7)	63±13 (7)	<.003
	6-7	6998±5515 (5)	98±14 (7)	<.001
	8-10	13712±9147 (11)	79±14 (12)	<.001
Anti-Histone (Ig) <sup>a</sup>	<2	741±264 (7)	52±8 (7)	<.001
	3-4	837±436 (7)	53±14 (7)	<.003
	6-7	4220±933 (5)	60±10 (7)	<.001
	8-10	16555±4618 (11)	295±173 (12)	<.001

5 <sup>a</sup> includes two weak positive.

<sup>\*</sup> Weak positive

a: Data is shown as number of ANA positive (mean±2sd of transgene negative littermates) mice using ANA screen kit

b: Data is represented as mean±SE for each group. Values are shown as Units/mL

10 NS: not significant

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#### Abbreviations

Abbreviations as used throughout this specification are defined as follows, unless otherwise defined in specific instances.

5	CDR	complementarity determining region
	EST	expressed sequence tag
	ORF	open reading frame
	SDS	sodium dodecyl sulfate
	TNF	tumor necrosis factor

10

While the present invention has been described in terms of the preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the

15 appended claims cover all such equivalent variations that come within the scope of the invention as claimed.

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What is claimed is:

1. An isolated or recombinant polypeptide having a sequence comprising SEQ ID NO: 25.
2. The polypeptide of Claim 1, further comprising SEQ ID NO: 26.
- 5 3. The polypeptide of Claim 1 having a sequence comprising SEQ ID NO: 27.
4. The polypeptide of Claim 1 having a sequence comprising SEQ ID NO: 5.
5. A polypeptide of Claim 1 capable of eliciting B cell growth, survival, or  
10 activation in mesenteric lymph nodes.
6. The polypeptide of Claim 5, wherein the sequence comprises SEQ ID NO: 2.
7. The polypeptide of Claim 5, wherein the sequence comprises SEQ ID NO: 4.
- 15 8. The polypeptide of Claim 5, wherein the sequence further comprises the C' through I region of SEQ ID NO: 6.
9. An isolated nucleic acid encoding a protein of any of Claims 1 to 8.
10. The nucleic acid of Claim 9 including one or more codons preferred for Escherichia coli expression.
- 20 11. The nucleic acid of Claim 9 having a detectable label attached thereto.
12. An expression vector comprising the nucleic acid of Claim 9.
13. An expression vector comprising the nucleic acid of Claim 10.
14. A host cell transformed or transfected with the expression vector of Claim 12.
- 25 15. A host cell transformed or transfected with the expression vector of Claim 13.
16. The host cell of Claim 14, wherein the cell is a prokaryotic cell.
17. The host cell of Claim 15 wherein the cell is a prokaryotic cell.
18. The host cell of Claim 16, wherein the cell is Escherichia coli.

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19. The host cell of Claim 17, wherein the cell is Escherichia coli.
20. A process for producing an AGP-3 protein comprising:
- (a) growing under suitable nutrient conditions host cells transformed or transfected with the nucleic acid of Claim 9; and
  - 5 (b) isolating the polypeptide product of the expression of the nucleic acid.
21. A polypeptide produced by the process of Claim 20.
22. The protein of any of Claims 1 to 8, wherein the protein has been covalently modified with a water-soluble polymer.
- 10 23. The protein of Claim 22 wherein the polymer is polyethylene glycol.
24. An antibody or fragment thereof which specifically binds the polypeptide of Claim 1.
25. The antibody of Claim 24 which is a monoclonal antibody.
26. A method for detecting the presence of an AGP-3 related protein in a
- 15 biological sample comprising:
- (a) incubating the sample with the antibody of Claim 24 under conditions that allow binding of the antibody to the AGP-3 related protein; and
  - (b) detecting the bound antibody.
- 20 27. A method to assess the ability of a candidate compound to bind to an AGP-3 related protein comprising:
- (a) incubating the AGP-3 related protein with the candidate compound under conditions that allow binding; and
  - (b) measuring the bound compound.
- 25 28. The method of Claim 27 wherein the compound is an agonist or an antagonist of an AGP-3 related protein.
29. A method of regulating expression of an AGP-3 related protein in an animal comprising administering to the animal a nucleic acid complementary to the nucleic acid of Claim 9.

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30. A pharmaceutical composition comprising a therapeutically effective amount of a protein of Claim 1 in a pharmaceutically acceptable carrier, adjuvant, solubilizer, stabilizer and/or anti-oxidant.
31. A method of modulating B cell growth, survival, or activation in a  
5 manual comprising administering a therapeutically effective amount of a modulator of the protein of Claim 1.
32. The method of Claim 31 wherein the modulator is an antibody, or fragment thereof, which specifically binds an AGP-3 related protein.

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## TNF-RELATED PROTEINS

### Abstract

A novel member of the tumor necrosis factor family is described. This  
5 member is primarily expressed in B cells and its expression correlates to  
increases in the number of B cells and immunoglobulins produced. The  
natural, preferred human ortholog is here called TBAF (TNF family B cell  
Activation Receptor) or AGP-3. The human ortholog contains 285 amino  
acids; the mouse ortholog, 309 amino acids. The protein is a type II  
10 transmembrane protein and has an amino terminal cytoplasmic domain, a  
transmembrane domain, and a carboxy terminal extracellular domain.  
TNF-related proteins of the invention may be membrane-associated or in  
soluble form, recombinantly produced or isolated after natural  
production. The invention provides for nucleic acids encoding such TNF-  
15 related proteins, vectors and host cells expressing the polypeptides, and  
methods for producing recombinant proteins. Antibodies or fragments  
thereof that specifically bind the proteins are also provided.

FIGURE 1

Human AGP3

10 30 50  
 GAATTCGGCAGCTGAGGGGTGAGGCAAGCCCTGCCATGTAGTGCAAGGACATCA  
 70 90 110  
 ACAAACACAGATAACAGGAAATGATCCATTCCCTGTGGTCACCTATTCTAAAGGCCCAA  
 130 150 170  
 CCTTCAAAAGTTCAAGTAGTGATATGGATGACTCCACAGAAAGGGAGCAGTCACGCTTAC  
 M D D S T E R E Q S R L T  
 190 210 230  
 TTCTTGCCTTAAGAAAAGAGAAGAAATGAACTGAAAGGAGTGTGTTCATCCCTCCACG  
 S C L K K R E E M K L K E C V S I L P R  
 250 270 290  
 GAAGGAAAAGCCCTCTGTCCGATCCTCCAAAGACGGAAAAGCTGCTGGCTGCAACCTTGCT  
 X E S P S V R S S K D G K L L A A T L L  
 310 330 350  
 GCTGGCACTGCTGTCTTGTGCTCAAGGTGGTGTCTTCTACCAGGTGGCCGCTGCA  
 L A L L S C C L T V V S F Y Q V A A I Q  
 370 390 410  
 AGGGGACCTGGCCAGCCTCCGGGACAGCTGCAAGGCCACCGGAGAAAGCTCCAGC  
 G D L A S L R A E L Q G H H A E K L P A  
 430 450 470  
 AGGAGCAGGAGCCCCAAGGCCGGCTGGAGGAAGCTCAGCTGTTCACCGCGGACTGAA  
 G A G A P K A G L E E A P A V T A G L K  
 490 510 530  
 AATCTTTGAACCAACAGCTCCAGGAGAAGGCAACTCCAGTCAGAACAGCAGAAATAAGCG  
 I F E P P A P G E G N S S Q N S R N K R  
 550 570 590  
 TGGCGTTCAAGGTCCAGAAAGAAACAGTCACTCAAGACTGCTTGCAACTGATTGCAGACAG  
 A V Q G P E E T V T Q D C L Q L I A D S  
 610 630 650  
 TGAACACCAACTATACAAAAAGGATCTTACACATTTGTTCCATGGCTCTCAGCTTTAA  
 E T P T I Q K Q S Y T F V P W L L S F K  
 670 690 710  
 AAGGGGAAGTGCCTAGAAAGAAAAGAGAATAAATATTGGTCAAGAAACTGGTTACTT  
 R Q S A L E E K E N K I L V K E T O Y F  
 730 750 770  
 TTTTATATATGGTCAGGTTTATATATAGATAAGACCTACGCCATGGGACATCTAATCA  
 F I Y G Q V L Y T D K T Y A M G H L I Q  
 790 810 830  
 GAGGAAGAAAGTCCATGTCTTTGGGGATGAATTGAGTCTGGTGACTTGTTCGATGTAT  
 R K K V H V F G D E L S L V T L F R C I  
 850 870 890  
 TCAAAATATGCTGAAACACTACCAATAATTCTGCTATTTCAGCTGGCATTGCAAACT  
 Q N M P E T L P N N S C Y S A G I A K L  
 910 930 950  
 GGAAGAGGAGATGAACCTCAACTTCAATAGCAAGAGAAAATGCACAAATATCACTGGA  
 E E G D E L Q L A I P R E N A Q I S L D  
 970 990 1010  
 TGGAGATGTCACTTTTGGTGCAATTGAAACTGCTGTGACCTACTTACACCATGTCTGT  
 G D V T F F G A L K L L  
 1030 1050 1070  
 AGCTATTTCTCCCTTTCTCTGTACCTCTAAGAAAGAAAGAACTAACTGAAAAATACAA  
 1090 1110 1130  
 AAAAAAAAAAAAAAAAAAGTAGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  
 1150 1170  
 AAAAAAAAAAAAAAAAAACTCGGAGGGGG



### Mouse AGP3

10 30 50 70 90 110 130 150 170 190 210 230 250 270 290 310 330 350 370 390 410 430 450 470 490 510 530 550 570 590 610 630 650 670 690 710 730 750 770 790 810 830 850 870 890 910 930 950 970 990 1010 1030 1050 1070 1090 1110 1130 1150 1170 1190

QAATTGGCACGAGCTCCAAAGGCGTACACCTTCAAAGTGCTCTCGTGGAAATGGATGAG  
M D E

TCTGCAAAAGCCCTGCCACCAACGCTGCTCTGTTTCTCCGAGAAAGGAGAGATATG  
S A K T L P P P C L C F C S E K G E D M

AAAGTGGGATGATGCCATCACTCCGACGAAGGAGGAGGGTGCCTGTTTGGGATCGC  
K V G Y D P I T P Q K E E G A W F G I C

AGGGATCGAAGGCTGCTGGCTGCTACGCTCTCTGCTGGCCCTGTTGTCCAGCAGTTTCA  
R D G R L L L A A T L L L A L L S S S S F T

GGGATGTCTTGTACCACTTGCTGCCCTTCCAAAGCAGACCTGATGAACCTCGCGCATGGAG  
A M S L Y O L A A L Q A D L M N L R M E

CTGCAGAGCTACCGAGGTTTACGAACACACGCGCCGCGGTGCTCCAGAGTTGACCGCT  
L Q S Y R G S A T P A A A G A P E L T A

GGAGTCAAACCTCTGACACCGCGAGCTCTCGACCCCAACAATCCAGCCGCGCCGACAGG  
G V X L L T P A A P R P H N S S R G H R

AACACGACGCGCTTCCAGGGACGAGGAAACAGAAACAAGTGTAGACCTCTCACTGCTCT  
N R R A F Q G P E E T E Q A D V D L S A P

CCTGCACCATCGCTCTGGATGGCGCATCTCAACATGATGATAATGGAATGAACCTC  
P A P C L P G C R H S Q H D D N G M N L

AAAGAACATCAATAGACTGTCTGACGATGATGACAGCAGCGACCGCACTATACGA  
R N I I Q D C I Q L I A D S D T P T I R

AAAGGAACTTACACATTGTTCATGCGCTTCTACGCTTAAAGAGGAAATGCCTTGGAG  
K G T Y T F V P W L L S F K R Q N A L E

GAGAAAGAGAAACAAATAGTGGTGGGCAACAGGCTATTTCTTCATCTACAGCAGGTT  
E X E N K I V I V T Q T G Y F F I S Q V

CTATACACGGACCCCATCTTGTCTATGGGTCACTCATCCAGAGGAAAGATACAGCTC  
L Y T D P I F A M G H V I Q R K E V H V

TTGGGGACGAGCTGAGCTCTGGTACCGCTGTTCGATGTATTGAGAATATGCCAAAAACA  
F G D E L S L V T L F R C I Q N M P K T

CTGCGCAACAATCTCGTCACTTGGCTGGCATCCGAGCTGGAAAGAGGATGGATT  
L P N N S C Y S A T G I A R L E E G D E I

CAGCTTGCAAATTCCTCGGAGAAATGCCACAGATTCAACGCAACGGAGACAGACCTCTTT  
Q L A I P R E N A Q I S R N G D D T F F

GGTGCCTTAAACTGCTGTAACTCACTTCTGCTGGATGCTGTATCCCTTCCCTCTGCTTC  
G A L K L L

TCTGTACCTCCGAGGGAGAAACAGACGACTGGAAAAATAAAAGATGGGGAAAAAGCCGTCA  
1090 1110 1130

GCGAAAGTTTCTCTGTGACCGGTTGAATCTGATCCAAACACAGGAAATATAACAGACAGCC  
1150 1170 1190

FIGURE 3

Alignment of human and murine AGP3 protein sequences

Hagg3	MDDSTEREQ SRLTSCICKR EEMKLECVS ILFRKESPSV RSSKDGLLA
Magg3	MDESAKTLPP PCLCPSEKGE DMKVGVDPI TPQKEGAWF GICRDGRLLA
cons	MD.S _____ L C K E MK _____ E. _____ DG.LLA
Hagg3	ATLLALLSCCLTVYSEYOV AALQGDLSL RAEIQGHAE KLPAGAGAPK
Magg3	ATLLALLSS SPTAMSLYOLAALQADLMNL RMELQSYRGS ATPAAAGAPE
cons	ATLLALLS. _ T _ S. YQ.AALQ. DL _ L R. ELQ _ PA. AGAP.
Hagg3	AGLEEAFAVT AGLKIFETPA PGEINSSONS RNKRAVQGP E ET _ _ _
Magg3	LT AGVKLLTPAA PRPHNSSRGH RNRRAFQGP E ETEQDVLDA
cons	_____ T AG. K _ P. AP _ NSS _ RN. RA. QGPE ET
B	
Hagg3	_____ VTQDCLQ LIADSETPTI QKGSYTFVPW
Magg3	TPAPCLPGCR HSQHDDNGMN LNNIQDCLQ LIADSDTPTI RKGTYTFVPW
cons	_____ QDCLQ LIAD.TPTI _ .KG.YTFVPW.
B' C C' D' E	
Hagg3	LLSFKRGSALE EKENKILVK ETGYFFHYGO VLYTDKTYAM GHLIQRKKVH
Magg3	LLSFKRGSALE EKENKIVVR QTGYFFHYGO VLYTDPIEAM GHVIQRKKVH
cons	LLSFKRGSALE EKENKILV. .TGYYFFHY.Q VLYTD. _ .AM GHLIQRKKVH
F G H	
Hagg3	VFGDELSLVT LFRCIQNMP E ILPNNSCYSA GIAKLEEGDE LQLAIPRENA
Magg3	VFGDELSLVT LFRCIQNMPK ILPNNSCYSA GIAKLEEGDE LQLAIPRENA
cons	VFGDELSLVT LFRCIQNMP. ILPNNSCYSA GIALEEGDE.QLAIPRENA
I	
Hagg3	QISLDGDDVTE FGALKLL
Magg3	QISRNGDDTE FGALKLL
cons	QIS. QD.TF FGALKLL

FIGURE 4

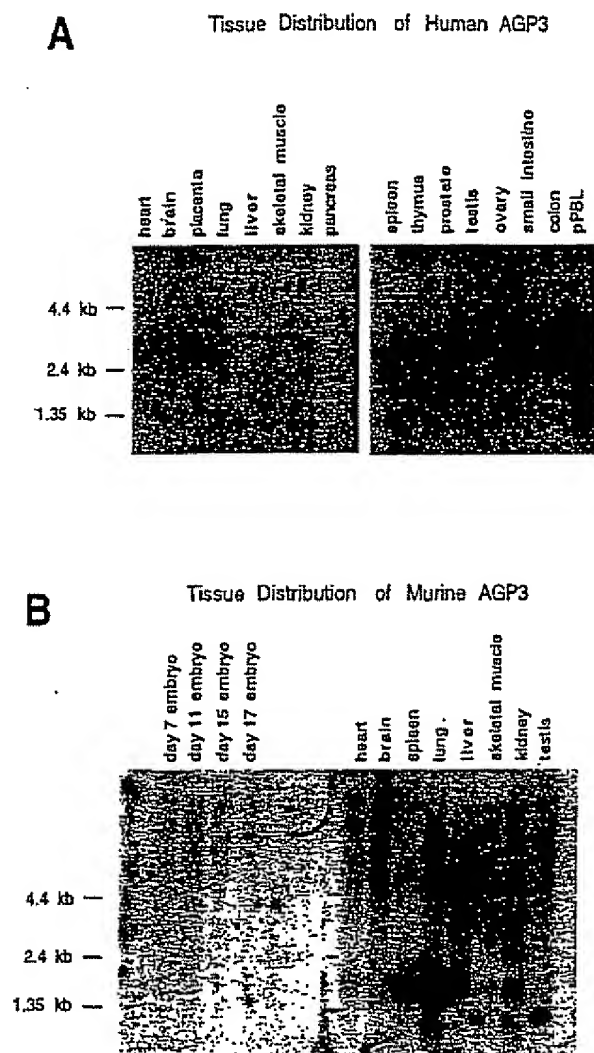


FIGURE 5

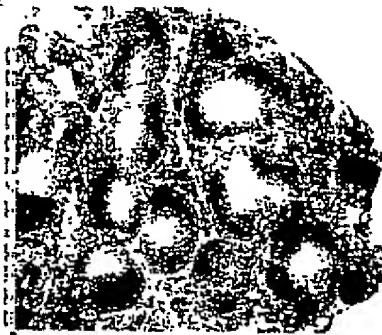


A

98M297 HH5 - control spleen, H&E, 2x



98M297 HH5 - expressor spleen, H&E, 2x



98M297 HH5 - control spleen, B220, 2x



98M297 HH5 - expressor spleen, B220, 2x



E

98M297 HH5 - control spleen, CD3, 2x



98M297 HH5 - expressor spleen, CD3, 2x

FIGURE 6



A  
98M297 HH5 - control lymph node H&E, 4x



B  
98M297 HH5 - expressor lymph node H&E, 4x



C  
98M297 HH5 - control lymph node B220, 4x



D  
98M297 HH5 - expressor lymph node B220, 4x

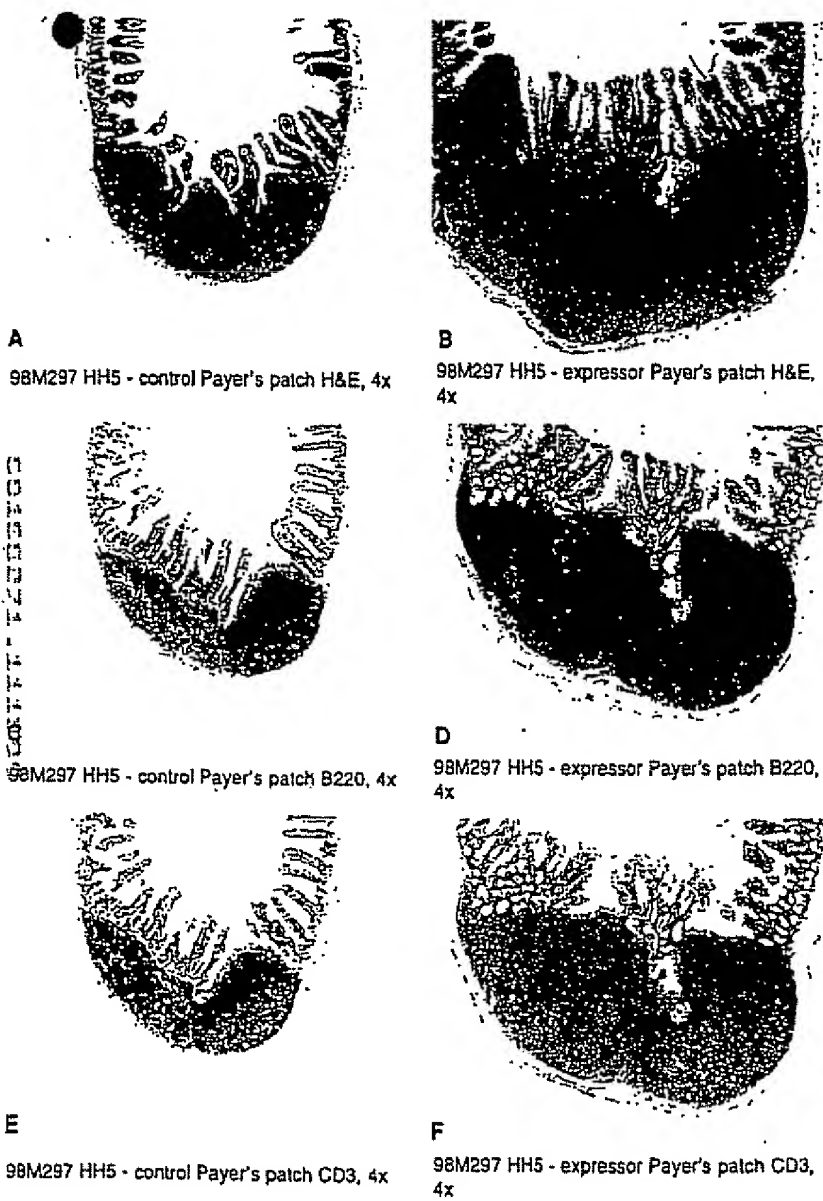


E  
98M297 HH5 - control lymph node CD3, 4x



F  
98M297 HH5 - expressor lymph node CD3, 4x

FIGURE 7



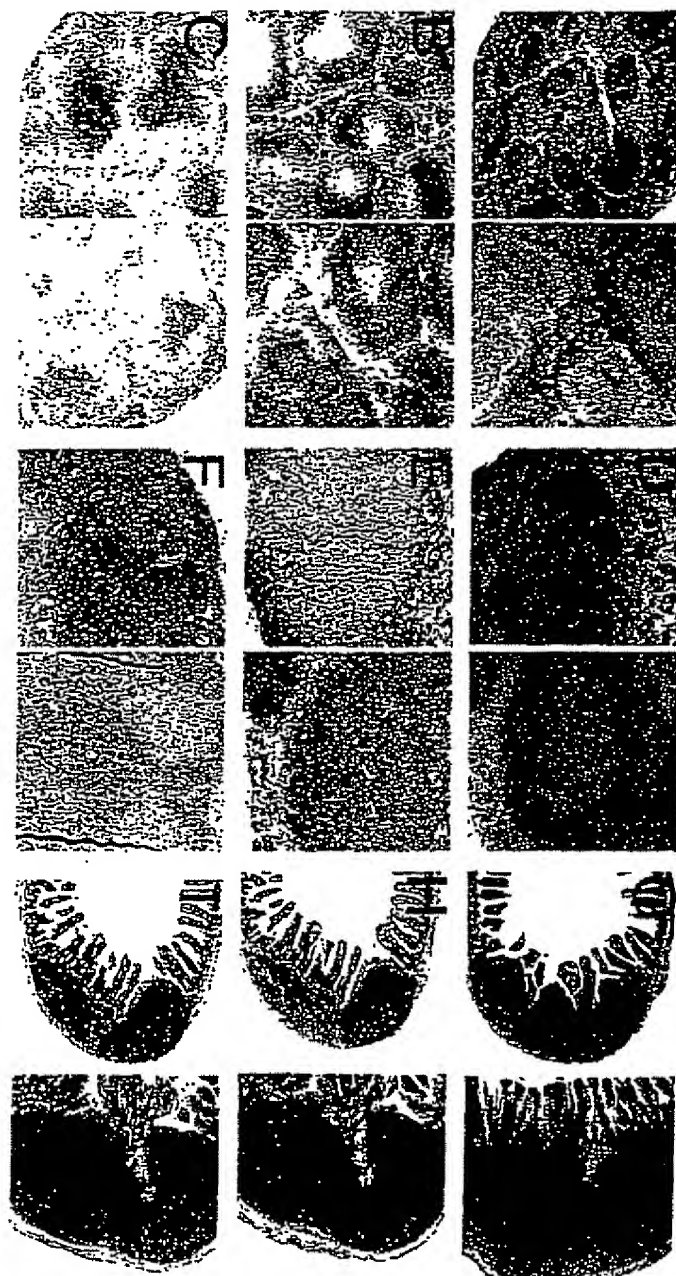


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139	137	136	116	142	153	137	135	112	110	97	82	82	85	200	202	205	190	189	121	212	213	216	213	214	213	210	201	101	102	161	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300
139	137	136	116	142	153	137	135	112	110	97	82	82	85	200	202	205	190	189	121	212	213	216	213	214	213	210	201	101	102	161	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300
139	137	136	116	142	153	137	135	112	110	97	82	82	85	200	202	205	190	189	121	212	213	216	213	214	213	210	201	101	102	161	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218																																																																																		



SECRET - 110000

FIGURE 10



SECRET - 110000

FIGURE 11

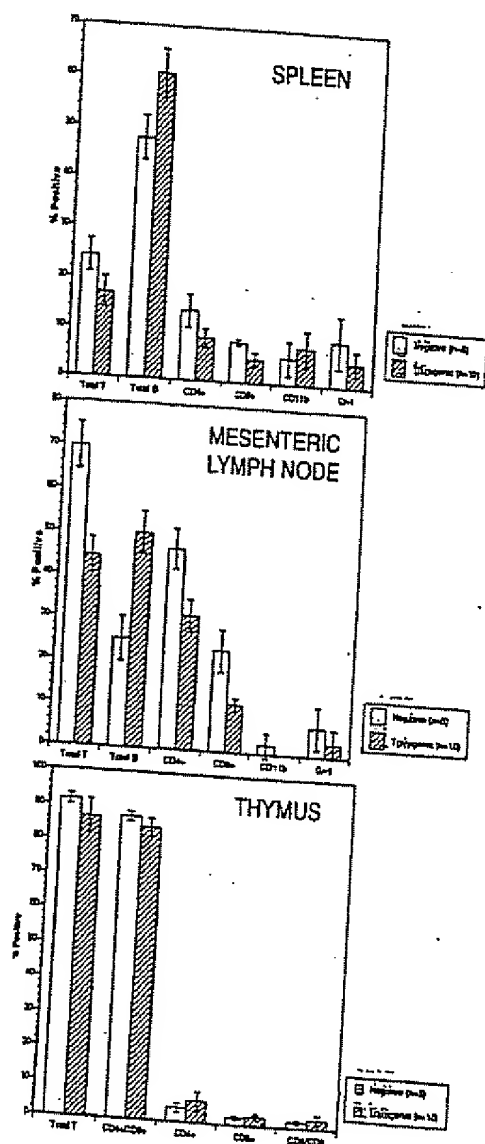
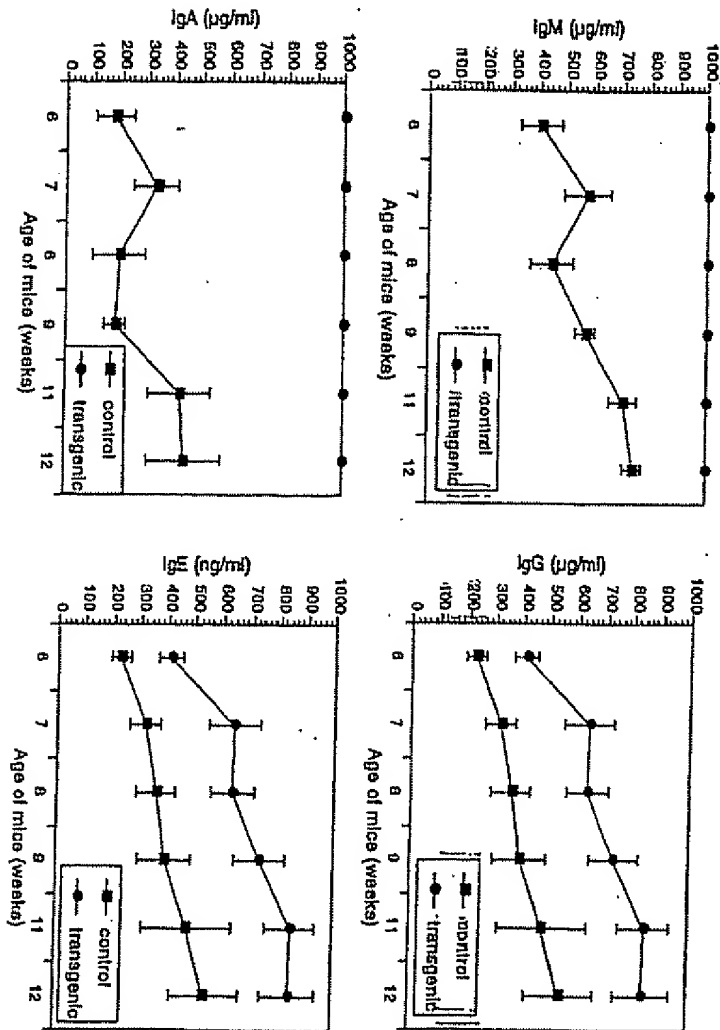
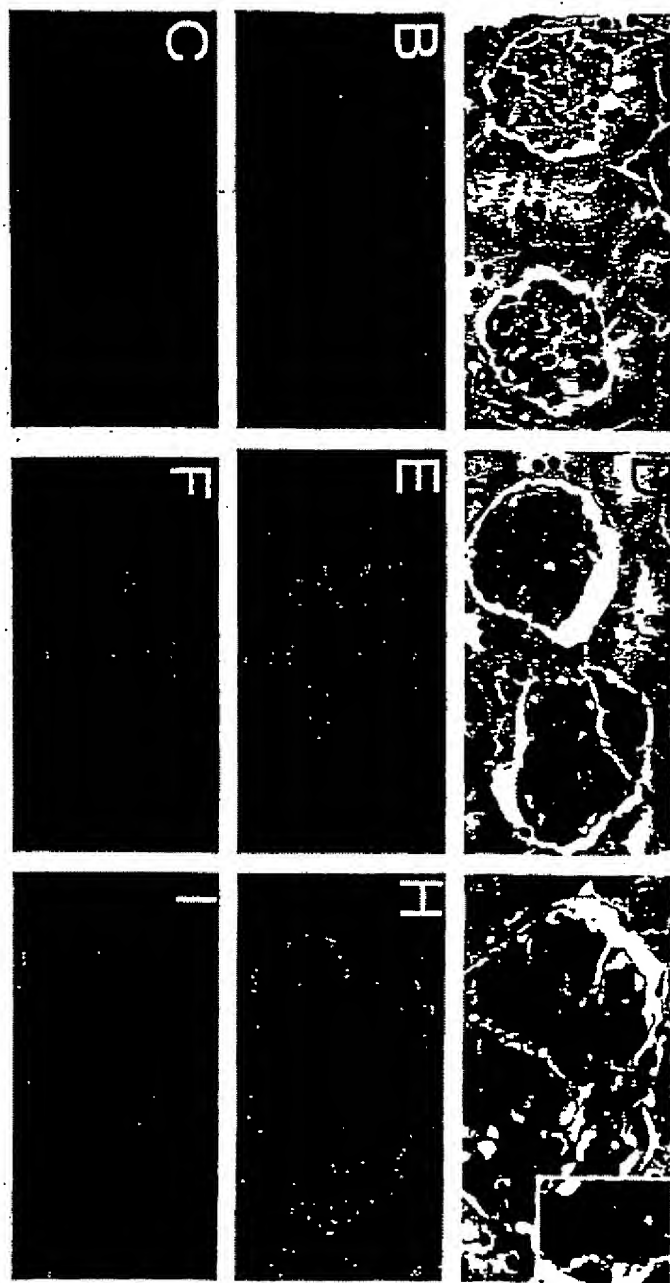


FIGURE 12



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FIGURE 13



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FIGURE 14

